THE RELATION OF SEASON, SEX AND WEIGHT TO THE BASAL METABOLISM OF THE ALBINO RAT

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The object of the present paper is to present data showing the effect of season, sex and weight upon the basal metabolism of the albino rat, and to compare the results computed as calories per kilogram per hour with those of calories per square meter per hour.

Sherwood, Savage and Hall ('33), Sherwood, Toth and Carr ('34), Sherwood and Bowers ('36), while investigating the effect of various substances upon the respiratory metabolism of the rat obtained approximately 6000 basal tests on both males and females over a period of 5 years. Out of this extensive data 1962 normal basal metabolism tests were obtained before the animals were used for experimentation. The normal tests represent data on seventy males and 100 females.

The presentation of the normal basal metabolism thus made possible seems of practical value since it may be used by future investigators for comparison with experimental data obtained during the various seasons.

EXPERIMENTAL PROCEDURE

The animals were fed a well-balanced dry ration (dog chow)[1] which was supplemented with fresh whole milk and green plant material. They were kept in an environmental temperature which varied within the limits of 21°C. to 29°C., the average temperature being approximately 26°C.

[1] Purina brand.

The apparatus used was a modification of the Haldane open circuit type (1892) which has been successfully used for small animals such as the rat, mouse, rabbit and guinea pig. The apparatus was kept in a room of thermic neutrality (28°C.) where the basal metabolism determinations were made.

Horst, Mendel and Benedict ('34 a) found that activity increased the basal metabolism in young males per unit of surface but not per unit of body weight. The rats used in the present investigation were kept in the same type of cage the year round and were therefore not exposed to an exercise variable. The hours between 10 A.M. and 4 P.M. as shown by Horst, Mendel and Benedict ('34 b) were found to be the most desirable for metabolism experiments since the animals usually slept during the major portion of the 3-hour testing period. It was found that when slight movement occurred normal heat production was not disturbed, which is in confirmation of the report of Benedict and MacLeod ('29 a).

The animals soon learned to adjust themselves to the 18-hour fast and regained the weight lost during the beginning tests. Probably due to their inability to withstand the fasting periods, the younger rats weighing less than 100 gm., did not give the consistent results obtained in the higher weight groups.

The heat production was calculated for surface area (Diack, '30) as well as for weight. During a preliminary investigation of 6 months to find the most suitable testing period, 2-, 3-, 4- and 6-hour periods were used. The 2-hour period did not give a sufficient change in weight of the apparatus for reliable measurement. The increased activity of the rats during the 4- and 6-hour periods seemed to interfere with the normal basal metabolism. The 3-hour period was therefore used for each basal metabolism determination.

More than 3500 normal basal metabolism determinations were obtained. However, nearly one-half of these were discarded since they followed experimentation. Only data were used that were known to be normal in every respect.

Benedict and MacLeod ('29 b) considered nineteen tests covering a period of 6 months detrimental to the health of the animal and, therefore, disturbing to the basal metabolism. Many rats in this laboratory have had basal metabolism tests every other day for a period of 6 months and no ill effects were observed. The weight was kept constant and the heat production did not vary from the normal. One female was used successfully from January to January of the next year and gave normal results when compared with the control rats of the various seasons.

RESULTS

Table 1 represents data obtained upon seventy males weighing from 100 to 420 gm. In the table are presented both the weight and surface area calculations for the sake of comparison. The actual metabolism averages for the weight groups as well as for the months are given. The number of tests for each weight group and each month are also presented.

Where the data are sufficient, as in the group of animals weighing from 141 to 260 gm., there is some indication of a seasonal variation. The heat production is slightly lower during the summer months. This picture is less pronounced in the males than in the females.

During the month of February the data are sufficient to show the effect of weight upon the basal metabolism. The surface area data show a difference of 26% in heat production between the rats weighing 100 gm. and those weighing 420 gm. The data calculated as calories per kilogram per hour for the month of February show a 42% difference in the weight range mentioned above. The tendency for a decrease in heat production as the animals increase in weight is indicated more or less for all other months.

In table 2 the data represent the metabolism to the extent of 1259 tests obtained upon 100 females ranging in weight from 100 to 420 gm. The data are presented in the same manner as shown for the males. There is no indication of a seasonal variation in the animals weighing less than 181 gm.

TABLE 1

Basal metabolism of the male rat

	GRAMS								TOTAL TESTS	ACTUAL AVERAGE
	100–140	141–180	181–220	221–260	261–300	301–340	341–380	381–420		
Calories per square meter per hour										
January	49.7	41.5	37.3	43.6	39.5				83	42.2
February	46.9	45.5	39.5	39.9	39.6	42.5	36.3	34.4	127	40.9
March		43.8	40.6	33.3	37.7	37.4	34.3		87	38.6
April		40.9	37.7	37.0	34.2				78	38.2
May	43.6	39.7	32.6	37.9					57	38.2
June		38.1	37.1	36.4					49	37.2
July		39.6	36.5	31.7					16	38.9
August		37.5	35.8	37.1	39.0				29	37.7
September		37.8	39.8	42.8	40.1				13	42.0
October		39.5	43.7	40.3					27	40.7
November	38.7	38.8	38.2	39.9	37.5				56	38.6
December	41.0	37.5	39.9	31.8					81	38.4
									(703 total)	
Number tests	40	205	190	181	60	7	16	4		
Actual average	46.2	40.7	38.3	40.2	31.5	40.3	35.4	34.4		
Calories per kilogram per hour										
January	7.38	5.85	5.34	5.22	4.57				83	5.87
February	6.90	6.29	5.05	4.79	4.61	4.70	3.88	4.00	127	5.15
March		5.93	5.17	4.38	4.37	4.17	3.70		87	4.71
April		5.60	4.82	4.40	3.89				78	4.88
May	6.38	5.46	5.13	4.33					57	5.38
June		5.25	4.68	4.12					49	4.68
July		5.36	4.49	3.80					16	4.82
August		5.27	4.72	4.67	4.50				29	4.75
September		5.52	5.04	5.20	4.69				13	5.17
October		5.36	5.32	4.88					27	5.16
November	5.63	5.16	4.27	4.90	4.30				56	4.97
December	5.94	5.10	5.29	4.47					81	5.20
									(703 total)	
Number tests	40	205	190	181	60	7	16	4		
Actual average	6.82	5.58	5.08	4.86	3.64	4.47	3.80	4.00		

TABLE 2

Basal metabolism of the female rat

	GRAMS								TOTAL TESTS	ACTUAL AVERAGE
	100–140	141–180	181–220	221–260	261–300	301–340	341–380	381–420		
Calories per square meter per hour										
January	42.3	39.3	39.7	38.6	36.8	36.0	38.8		157	39.1
February	41.3	40.7	40.0	31.5	39.3	35.2	30.8		209	39.1
March	40.0	39.5	40.8		35.3	37.1			196	39.2
April	39.9	40.6	43.1	38.6					177	40.9
May	44.7	40.3	38.0	41.8					102	40.5
June	37.6	38.9	36.7						21	37.6
July	41.4	39.3	37.5	39.2	39.4	35.2			41	38.3
August	38.9	42.5	38.4	37.5	37.3				106	39.0
September	45.9	41.0	43.6						52	40.7
October	41.9	41.3	43.6						58	42.2
November	37.3	38.1	38.3						52	38.0
December	42.2	39.4	38.7						87	39.8
									(1259 total)	
Number tests	117	590	369	31	101	49	2			
Actual average	41.1	40.2	39.8	37.7	36.6	35.9	34.8			
Calories per kilogram per hour										
January	6.23	5.43	5.12	4.79	4.28	3.79	4.15		157	5.07
February	6.04	5.59	5.20	3.76	4.20	3.88	3.29		209	5.06
March	5.87	5.34	5.18		3.97	4.08			196	5.11
April	5.74	5.53	5.61	4.76					177	5.47
May	6.73	5.52	4.96	5.13					102	5.54
June	5.55	5.21	4.74						21	5.05
July	6.56	5.34	4.81	4.59	4.57	3.94			41	5.00
August	5.94	5.89	4.91	4.66	4.33				106	5.02
September	6.66	5.68	5.05						52	5.37
October	6.06	5.68	5.65						58	5.68
November	5.50	5.08	4.96						52	5.18
December	6.23	5.36	5.05						87	5.44
									(1259 total)	
Number tests	117	590	369	31	101	49	2			
Actual average	6.07	5.48	5.12	4.59	4.19	3.90	3.72			

The weight group from 181 to 220 gm. demonstrates a definitely lower heat production during the summer months. However, the data are not sufficient to show this phenomenon in the larger and older animals. The females weighing 100 gm. show a 25.4% higher heat production than those weighing 380 gm. when calculated on the number of calories per square meter per hour. The calculations based on the calories per

TABLE 3

Basal metabolism of the rat

MALES			FEMALES		
Weight in grams	Calories kilo/hour	Calories sq.m./hour	Weight in grams	Calories kilo/hour	Calories sq.m./hour
131	6.63	45.1	131	6.77	45.3
136	6.37	43.9	136	6.10	42.0
146	6.17	43.3	146	6.07	42.5
151	5.87	38.1	151	5.70	40.6
158	5.23	37.8	158	5.38	39.0
168	5.29	38.9	168	5.39	41.5
170	5.33	39.6	170	5.22	39.1
175	5.13	39.2	175	5.19	38.9
179	5.03	38.2	179	5.03	38.0
189	5.20	39.8	189	5.17	39.8
197	4.60	38.8	197	4.98	39.0
211	4.53	38.6	211	4.82	38.3
220	4.90	39.5	220	4.93	39.9
226	4.74	38.9	226	4.64	38.0
247	4.69	39.3	247	4.48	35.0
264	4.52	39.0	264	4.01	34.4
277	4.62	39.9	277	4.30	37.6
289	4.17	37.3	289	4.05	36.0
302	4.15	37.2	302	4.00	35.6
304	4.18	37.5	304	3.84	34.5

kilogram per hour show a 45.5% difference in the same group of animals.

The coefficient of variability shows the males 7% more variable than the females when measured in calories per square meter. This means that on the average one male differs from another male 7% more than one female differs from another female when the variability is calculated on

the basis of calories per square meter. When measured, however, in calories per kilogram the males are only 5.4% more variable than the females. The heat production measured in calories per kilogram is more variable within both sex groups

TABLE 4

Respiratory metabolism of the female

	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES[1] IN 100
January	148	5.12	0.798			
				0.096	0.09	83[1]
February	163	5.03	0.903			
				0.087	0.06	76
March	246	5.09	0.798			
				0.064	0.47	100
April	216	5.56	0.580			
				0.065	0.07	85
May	148	5.49	0.626			
				0.110	0.50	100
June	39	4.99	0.607			
				0.146	0.00	50
July	29	4.99	0.584			
				0.124	0.29	99
August	105	5.28	0.632			
				0.091	0.10	86
September	77	5.38	0.590			
				0.099	0.09	82
October	54	5.29	0.538			
				0.102	0.13	89
November	67	5.16	0.582			
				0.095	0.36	100
December	100	5.52	0.632			

[1] "The chances of 83 in 100" means that if 100 groups of rats were taken during January and 100 groups during February, we would find that the differences between these groups would, in 83 cases, be as indicated in the table—in 17 of the 100 there would either be no difference or the difference would be in the opposite direction. Such chances are calculated on the assumption that the errors of measurement fall on a normal distribution curve.

than is that of the surface area metabolism. This fact is indicated by the standard deviations of the two groups.

Table 3 shows a comparison between animals of equal weight. Data were available on fifty females and fifty males. An average of ten tests on each animal shows a slightly

higher heat production in the male then in the female, in the larger animals. The younger animals have approximately the same basal metabolic rate.

Table 4 presents the standard deviation for each month for the females. The reliability of the difference from month to month is given in terms of the chances in 100 of there being

TABLE 5

Respiratory metabolism of the male rat

	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES IN 100
January	83	5.25	0.449			
				0.083	0.33	100
February	127	4.92	0.594			
				0.082	0.15	96
March	87	4.77	0.611			
				0.111	0.07	74
April	78	4.84	0.678			
				0.112	0.34	100
May	57	5.18	0.655			
				0.130	0.41	100
June	49	4.77	0.727			
				0.253	0.53	98
July	16	5.30	0.619			
				0.250	0.12	56
August	29	5.27	0.603			
				0.140	0.21	93
September	13	5.08	0.418			
				0.180	0.62	100
October	27	5.68	0.504			
				0.190	0.46	99.2
November	56	5.22	0.710			
				0.140	0.57	71
December	81	5.14	0.578			

a true difference in the metabolism from one month to the next. These chances were calculated on the assumption that the basal metabolism of the female albino rat followed the normal distribution curve and that adequate data were obtained on the basal metabolic rate. There is evidence that there is a true difference between the succeeding months of the year, except between June and July. Perhaps a reliable

TABLE 6

Respiratory metabolism of the male rat

WEIGHT IN GRAMS	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES IN 100
125–175	211	5.48	0.630	0.059	0.44	100
176–225	207	5.04	0.571			
176–225	207	5.04	0.571	0.051	0.32	100
226–275	196	4.72	0.445			
226–275	196	4.72	0.445	0.068	0.01	56
276–325	53	4.71	0.441			
276–325	53	4.71	0.441	0.117	0.71	100
326–400	25	4.00	0.500			

TABLE 7

Respiratory metabolism of the female rat

WEIGHT IN GRAMS	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES IN 100
125–175	685	5.63	0.662	0.035	0.46	100
176–225	540	5.17	0.573			
176–225	540	5.17	0.573	0.060	0.76	100
226–275	53	4.39	0.395			
226–275	53	4.39	0.395	0.069	0.39	100
276–325	94	4.00	0.414			
276–325	94	4.00	0.414	0.084	0.15	96
326–400	20	3.85	0.321			

difference would have been obtained if it had been possible to collect more data for these months.

Table 5 gives the comparable data for the male albino rat. The differences are clearly demonstrated except between July and August. An inspection of the averages reveals no definite seasonal curve. The seasonal effect is more pronounced in the females but the male basal metabolism shows greater variation.

Table 6 shows a decided decrease in metabolism as the males increase in weight except those weighing from 226 to 325 gm. Here the metabolism seems to remain rather constant until the rats reach a larger weight group where the drop is again very definite. In table 7 the females show a decided decrease for every group as the weight increases. The critical ratio also becomes smaller as the animals increase in weight.

DISCUSSION

The data of this investigation were obtained from animals of the age commonly used in experimental laboratories. Since a survey of the basal metabolism of the albino rat seemed necessary it was decided to produce as complete a picture as possible by using both sexes and as many different weight groups as available.

A comparison of the data of the present investigation with those of Benedict and MacLeod ('29 b) shows the value of a greater number of basal metabolism tests for such an extensive problem as that undertaken by them. There is no indication of an increase in the basal metabolism with increasing age in the rats used in the present investigation as was reported by the above-mentioned authors who used animals as old and older than our larger weight groups. The most desirable animals for basal metabolism investigation were found in the weights ranging from 181 to 300 gm.

The heat production in the control rats reported by Horst, Mendel and Benedict ('34 a) was slightly higher than that found in the present investigation. Horst, Mendel and Benedict ('34 b) found the hours 10 A.M. to 4 P.M. suitable

for measuring the heat production. In the present investigation it was noticed that the rats usually slept during the 3-hour testing period. When the animals were excessively active the tests were discarded.

Upon examination of the data of the above authors one sees a decrease in metabolism with increasing age in both males and females which is in agreement with the data of the present investigation. However, the statement is made that males have a higher heat production than females. Rat no. 3 of their data, a male weighing 137 gm., shows a heat production of 27.2 cal. per 200 gm. per 24 hours compared with rat no. 9, a female, weighing 139 gm., 30.4 cal. per 200 gm. per 24 hours. This male is younger and should therefore have a much higher heat production. Female no. 2 of the same data shows a heat production of 27.3 cal. compared with the lower heat production of 22.2 cal. of a male weighing 239 gm., the female in this case weighing 236 gm. The data seem inadequate for a comparison of sexes and the number of females, six animals, not sufficient for comparison with the males, fifty-four animals.

Horst, Mendel and Benedict ('34 b) state that rats weighing from 100 to 320 gm. have essentially the same metabolism at 30°C. when measured per unit of surface area, in reviewing the work of Houssay and Artundo ('29) but fail to mention that these authors show a heat production of 8.2 cal. per kilogram per hour in rats weighing from 100 to 139 gm. and a heat production of 5.7 cal. per kilogram per hour for rats weighing from 280 to 320 gm. This is a reduction of approximately 30% in heat production from the smaller to the larger animals, a phenomenon which is certainly worthy of mention. In the present investigation there was a decrease in heat production of approximately 26% when calculated per unit of surface area in the weight range from 100 to 420 gm. and a 45% decrease in the same weight range when measured per kilogram per hour. The metabolism of the rats mentioned by Houssay and Artundo ('29) is somewhat higher at 30°C. than that found in the present

investigation. This may have been due to the fact that our animals were kept in a room where the average yearly temperature was between 25°C. and 29°C. thereby causing a lower heat production.

Benedict and MacLeod ('29 b) show a seasonal variation in the basal metabolism of the albino rat but do not take into consideration size, sex and age effects with respect to seasonal heat production. The present investigation does not show a seasonal variation in all weight groups. It appears in animals weighing above 180 gm. but below that weight there is no indication of a seasonal influence in either sex. It may be that in young growing rats the seasonal variation is obliterated by the higher heat production and constant change in weight.

Gustafson and Benedict ('28) attempt to show a seasonal variation in the basal metabolism of young women. Unfortunately they present no data for the months of July, August and September which seem important for a complete seasonal picture.

Mitchell and Carman ('26) report a decrease in heat production with an increase in weight and age in younger animals. They show that the basal metabolism of male rats per unit of body surface area or of body weight is more variable than that of female rats. In both sexes the variability of the basal metabolism referred to body surface was less than that referred to body weight.

With so many cases available, it was thought that a comparison of the two methods used in calculating heat production would be of value. That the two methods do not measure the same thing is evident since the coefficients of correlation are 0.84 and 0.71 for the females and males respectively. These coefficients are high, but since they have been corrected for sampling errors, one would expect a coefficient of 0.90 or above if they were equally valid.

A word might be said about the meaning of coefficients of correlation. The range of the coefficient is from plus one to minus one. A coefficient of plus one indicates that the two series of measurements are so closely related that one may

be used as an indication of the other. It does not mean, however, that the one is the cause of the other. The coefficients of 0.84 and 0.71 obtained in the present investigation indicate, therefore, that the two technics used in calculating heat production do not give identical results. That is, we cannot use one to predict, with perfect reliability, the other.

The data also afford some information as to the reliability of the two technics. By perfect reliability it is meant that the technic applied on two different occasions to the same organism under the same conditions will give the same result. Consequently, the metabolic reading obtained on 2 successive days, on the same rats were correlated. Surface area measurements were as follows: For females 0.83, for males 0.79. Measurements per kilogram were 0.88 for females and 0.83 for males. If the technics were perfectly reliable, the coefficients would have been plus one. We may conclude from these coefficients that the weight technic is slightly more reliable. However, this might not have been the case if other surface area formulae had been used in the calculations.

In spite of the slightly higher reliability of the weight technic, the surface area technic may be more valid. That is, it may measure more adequately, the daily heat production.

CONCLUSIONS

Adult rats weighing above 180 gm. show a slight seasonal variation in basal metabolism, being somewhat lower during the summer months.

The data show a decrease of 26% in heat production from animals weighing 100 gm. to those weighing 420 gm. when measured in calories per unit of surface area. A decrease of 42% is shown for the same group when measured in calories per kilogram.

The male has a more variable heat production than the female.

The basal metabolic rate is shown to be nearly the same in young males and females, to vary during active sexual life and finally to become nearly the same as the rats approach the end of sexual life.

The metabolism calculated as calories per square meter does not give the same picture as that calculated per kilogram of body weight.

The weight technic is slightly more reliable than the surface area technic as measured by Diack's formula.

The author wishes to express his appreciation for the helpful criticism given by Dr. Walter J. Meek in the method of presenting the material of the present investigation; to Dr. J. A. E. Eyster for his criticism of the statistical methods used in presenting the data; and to Dr. M. M. White for his most generous assistance with the statistical data.

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THE AVAILABILITY OF THE PROTEINS AND INORGANIC SALTS OF THE GREEN LEAF

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ONE FIGURE

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INTRODUCTION

The importance of vegetables in our diet has greatly increased in recent years due to the fact that attention has been centered upon the leafy foods as excellent sources of the 'little things in nutrition,' i.e., the vitamins and the inorganic salts. So extensive have been the investigations of these vital principles, that one probably knows much more about the composition of vegetables with respect to their content of vitamins and inorganic ions than about the amounts of digestible carbohydrate, fat and protein which they contribute in nutrition. While it is true that we do not ordinarily eat leafy vegetables as sources of energy, there are occasions when it is necessary to know very exactly the available protein, fat and carbohydrate content of these foods.

Most of the data on the composition of American food materials are based on Atwater and Bryant's famous bulletin no. 28 ('02). In this work, protein was determined by multiplying the total nitrogen of the foodstuff by a factor, 6.25.

[1] The data in this paper were taken from a dissertation presented by M. K. Horwitt in partial fulfillment of the requirements for the degree of doctor of philosophy, Yale University, 1935. Lack of space prevents the presentation of more detailed data than are reported here. Additional details are in the dissertation deposited in the Yale University Library.

A preliminary report of this material was presented before the American Institute of Nutrition, Washington, D. C., on March 25, 1936.

[2] Deceased December 9, 1935.

The inadequacy of this method has long been recognized. Not only is it true that all of the nitrogen is not protein nitrogen, but the use of the factor, 6.25, based on the fact that protein on an average contains 16% nitrogen, is often misleading. Jones ('31) has made a study of protein nitrogen and has recommended the use of different factors for different proteins.

The term 'fat' in food analysis is equivalent to the total ether extract. The ingredients of the 'ether-extract' of vegetable foods include along with the true fats and fatty acids various other substances such as lecithin, chlorophyll, xanthophyll and carotene. The pigments alone in some green leaves may account for as much as 50% of the 'ether-extract.' The inclusion of these indigestible residues as true fat may cause a large error when the caloric value of a food is determined.

The determination of total carbohydrate as ordinarily performed is probably the least accurate of all analyses conducted on foodstuffs. The reason for this lies in the fact that this proximate principle is estimated by difference, that is, the total per cent of the moisture, ash, protein and fat is substracted from 100% and the result called carbohydrate. Within recent years, because of the importance of preparing accurate food tables for diabetic diets, several methods have been suggested by which more accurate results may be obtained.

The fact that all the mineral constituents of vegetables are not available to the animal organism is not new. Little is known, however, concerning the amounts of the different mineral constituents which are assimilated.

At the beginning of the present study of the digestibility of leafy vegetables, it was our intention to use a variety of leafy foods in an attempt to obtain comparative results. When it became apparent that the methods available for food analysis as described in the literature were totally inadequate and that most of our time would have to be spent in the development of methods, it was deemed advisable to confine our studies, for the time being, to a single complex food substance.

It was believed that a thorough investigation of one food might be more satisfactory than a study of many foods in which less time could be spent on the different components of each.

Because of its cheapness and accessibility, and because it is representative of a type of vegetable about which there has been much discussion, spinach (the leaves of the plant, Spinacia oleracea) was chosen as the material for this study. It contains many of the substances which make difficult the analyses of vegetable foods, as well as a high nitrogen content and a representative amount of fat, carbohydrate and mineral matter.

Preliminary experiments with samples bought daily in the New Haven markets showed that the individual samples differed greatly from each other in composition. It was evident that if methods were to be developed, a material of more constant composition would have to be obtained. After due consideration a commercially dried spinach was selected, and a sufficient supply obtained and preserved in air-tight containers.

Some idea of the constancy of the composition of this dried spinach is gained from the fact that three separate samples of the mixed material averaged 6.45 ($\pm$ 0.03) % moisture, while the ash and nitrogen contents averaged 19.27 ($\pm$ 0.04) and 4.41 ($\pm$ 0.02) %, respectively, the latter two figures being expressed on a moisture-free basis.

In this paper and those which follow, the inadequacy of some of the methods applied in food analysis will be pointed out. An effort will be made to demonstrate the differences between total protein, fat and carbohydrate and the available foodstuff; these discrepancies are especially striking in the case of leafy foods. Wherever possible, availability was determined by means of in vitro digestion experiments.

PROTEINS

Ever since Stutzer (1880) applied the technic of in vitro digestion to protein analysis numerous attempts have been

made to determine the digestibility of different foods by estimating the increase in soluble nitrogen after digestion with pepsin or trypsin, or both. Since the literature on this topic is very extensive, it would not be practical to review the field in this paper. Some of the earliest applications of peptic digestion in the study of proteolysis are discussed by Berg ('09), but for the most part the results of the different investigators are difficult to compare because of the general lack of uniformity in the digestion methods employed.

Experimental

The laboratory experiments conducted on the in vitro digestion of the proteins of the green leaf can be classified under two main headings: a) Digestion studies in which the classical Kjeldahl nitrogen method is the criterion for the extent of digestion, and b) those in which the amino nitrogen liberated is determined.

a. Since other parts of the green leaf in addition to protein go into solution during a proteolytic digestion, it was thought that determinations of the total solids and total ash would be of value in the subsequent interpretation of the results. At first, the determination of the total solids presented a problem because of the difficulty encountered in filtering the digested mixture. After several unsuccessful trials in which Jena glass filters, Gooch crucibles and ordinary filter papers were used, it was found that the best results could be obtained with no. 40 Whatman filter paper in an ordinary filter funnel, if very slight suction was used during the filtration. By connecting a series of filter-flasks to a pump and by adjusting the extent of the vacuum to about 100 mm. of mercury, it was possible to get good suction and reduce to a minimum the chances of the filter paper tearing.

About 1 gm. of the dried spinach, accurately weighed, was transferred to a 50 cc. centrifuge tube. The digestive agent in 40 cc. of the appropriate solvent was then added to the spinach and thoroughly mixed. The solvents used were water, N/10 hydrochloric acid, and 0.5% sodium carbonate. Blank determinations were run parallel to the enzyme digestions for the full time interval of 24 hours for each. Where successive digestions were carried out, the hydrochloric acid

was filtered off at the end of 24 hours, before the digestion in the sodium carbonate was begun. To each tube, 0.2 cc. of tricresol was added as an antiseptic; then the tube was stoppered and incubated at 38°C. Several times during the day, the tubes were shaken by hand. At the end of 24 hours the separation of the liquid from the residue was begun. Each tube was centrifuged for 5 minutes at a moderate speed. Some of the residue floated on the surface, but the major portion of the digest was packed at the bottom of the tube. The liquid was carefully transferred to the weighed filter paper and filtered with the aid of slight suction. The residue in the centrifuge tube was then mixed with 40 cc. of distilled water and the process repeated until the solid had been washed a total of three times. At the end of the third washing the solid in the centrifuge tube was transferred quantitatively onto the same weighed filter paper. Air was drawn through this residue on the filter paper until it was almost dry; the filter paper plus the residue was carefully removed from the filter funnel and analyzed for total solids by drying in vacuo at 100°C. for 24 hours or more. After weighing, this dried material was incinerated, first over a free flame until thoroughly charred and then in an electric furnace at 550°C. until no carbon particles remained. The residue left after incineration was weighed and called the 'ash in residue.'

Nitrogen in the residue was determined on a different lot of spinach digestions by means of a Kjeldahl method.

Every digestion determination was carried out in duplicate.

The moisture, ash and nitrogen content of the enzymes[3] used were determined separately and proper correction made in each case. A summary of a typical series of digestions is given in table 1. The data in this table represent the averages of four determinations.

A preliminary experiment to determine whether the bacteria of the intestine have a digestive effect on vegetable matter was performed as follows:

Approximately 1 gm. of dried spinach was incubated with 40 cc. of an aqueous extract of human feces which had been filtered through a coarse filter paper. After 40 hours the

[3] The pepsin used was from the Wilson Laboratories, Chicago, Ill.; the trypsin was obtained from Fairchild Bros. & Foster, New York, N. Y. The manufacturers claimed it was eight times the strength of Pancreatin, U. S. P.

residues were washed and analyzed for solids, ash and nitrogen, by means of the technic described above. The results of this experiment are included in table 1.

Examination of table 1 shows that water alone takes out 43.7% of the total solids in the leaf, which means that almost one-half of the substance of the cell is soluble. Hydrochloric acid removes a little more than water, and sodium carbonate in turn removes slightly more than hydrochloric acid, or 52.6%. During digestion with pepsin and trypsin, each enzyme brought a little more solid into solution. The 67% figure obtained, when both pepsin and trypsin were used successively, is about 12% higher than the result of the control extraction with hydrochloric acid and sodium carbonate.

TABLE 1

Summary table of comparative digestions

DIGESTIVE AGENT	SOLIDS EXTRACTED	ASH EXTRACTED	NITROGEN EXTRACTED
	%	%	%
Extraction with water	43.7 ± 0.2	83.8 ± 0.1	36.4 ± 2.8
Extraction with N/10 HCl	46.4 ± 0.3	88.3 ± 0.1	35.0 ± 1.0
Extraction with Na_2CO_3 0.5%	52.7 ± 0.1	82.4 ± 0.4	53.4 ± 0.2
N/10 HCl followed by Na_2CO_3 (24 hours each)	55.1 ± 0.2	85.8 ± 0.2	44.2 ± 1.3
N/10 HCl plus 20 mg. pepsin	58.3 ± 0.2	88.7 ± 0.1	76.1 ± 0.4
Na_2CO_3 0.5% plus 50 mg. trypsin	61.8 ± 0.1	82.4 ± 0.4	84.5 ± 0.3
Pepsin followed by trypsin (24 hours each)	67.4 ± 0.1	88.8 ± 0.6	89.3 ± 1.2
Intestinal bacteria	46.0 ± 0.1	82.9 ± 0.4	44.8 ± 0.4

There are only slight differences between the amounts of ash extracted by the different agents.

Examination of the nitrogen data shows that less nitrogen was extracted by the hydrochloric acid than by any other solvent. This is to be expected because hydrochloric acid precipitates the protein present.

Proof that protein is extracted by water alone was obtained by acidifying a water extract of spinach; a copious precipitate formed which could be completely decolorized by washing in alcohol and acetone. This impure preparation gave protein color reactions, had a nitrogen content of 14% and could be coagulated by heat.

Sodium carbonate dissolved about 17% more nitrogen than water. This was not unexpected for both Osborne and Wakeman ('20) and Chibnall ('24) have shown that the protein of spinach is soluble in weak alkali. Pepsin alone made 76% of the nitrogen soluble in contrast to trypsin alone which brought 84.6% of the nitrogen into solution. That digestion studies of foods should be done by successive treatment with pepsin and trypsin is brought out by the fact that such treatment dissolved more nitrogen than the use of either of the enzymes individually.

The results of the experiment in which intestinal bacteria were the digestive agents show that there is not much difference between the effect of bacteria and the solvent action of pure water.

Approximately 90% of the nitrogen in spinach was made soluble by enzymatic activity. What is the nature of the other 10%?

Since chlorophyll and the cephalin-like compounds contain nitrogen and are soluble in ether, an experiment was performed to determine what percentage of the total nitrogen is in the ether extract. The results showed that about 5.67% of the total Kjeldahl-determined nitrogen is fat-soluble. The importance of this observation in food analysis, in which the nitrogen alone is the sole criterion of the protein content of the food, is self-evident.

b. Amino nitrogen studies. Studies of the in vitro digestion of proteins, in which one determines the amino nitrogen liberated, involve a technic entirely different from that just discussed.

A review of the development of methods for measuring amino nitrogen and the application of this to the study of the digestion of isolated proteins was made by Frankel in 1916. With this technic, it is possible to obtain an index of the extent of digestion of a protein beyond that stage in which the protein goes into obvious solution.

More recent investigations which apply the amino nitrogen liberation technic to the study of protein digestibility have

been made by Gabathules ('20), Greaves and Morgan ('34), Waterman and Johns ('21), Waterman and Jones ('21), Jones and Waterman ('22, '23) and Jones and Gersdorff ('33, '34).

If it is assumed that casein is a completely digestible protein, the digestibility of other proteins may be estimated by comparing the extent of their digestion in vitro with that of casein under similar conditions. An attempt was made to compare the protein of spinach with casein in order to test this technic.

The pepsin and trypsin used in these tests were identical with those described above. The erepsin was prepared according to a modification of the method used by Northrop and Simms ('28) as follows: Three portions of hogs' small intestine, each containing only the first 3 feet below the pyloric sphincter and obtained within a few hours after slaughter, were thoroughly ground and mixed with an equal volume of glycerol. Toluene was added and the mixture allowed to stand at room temperature for 2 days. This was then strained through cheese cloth and dialyzed in a cellophane tube against running water. After 12 hours dialysis, the mixture was filtered and the filtrate returned to a dialysis tube. After 8 hours of additional dialysis, the material was filtered and used.

The total amino nitrogen in the spinach was determined by hydrolyzing with 20% hydrochloric acid for 24 hours and analyzing the hydrolysates, after distilling off the HCl in vacuo, for amino nitrogen by the Van Slyke ('13) method; 5 gm. of dried spinach yielded 139.7 mg. In order to estimate how much amino nitrogen other than protein amino nitrogen was present in the dried leaf before it was hydrolyzed, 5 gm. samples of dried spinach were thoroughly extracted with 200 cc. of warm N/100 hydrochloric acid. The amino nitrogen in the extract was 14.6 mg.;[4] 139.7 mg. minus 14.6 mg. gives 125.1 mg. of amino nitrogen liberated from the protein of 5 gm. of dried spinach by acid hydrolysis.

Having estimated the total amount of amino nitrogen in the leaf, the amount which could be obtained by means of enzymatic hydrolysis was determined. All digestions were

[4] This figure does not include amino nitrogen from lecithin-like compounds which may be present in the leaf but this error is too small to affect the comparative results.

carried out in duplicate. Control experiments were run with all the reagents and enzymes in the same quantities as in the regular experiment except that no spinach was added. In each case, sufficient enzyme was used to give maximum digestion in terms of amino nitrogen liberated.

Twenty grams of spinach were mixed with 0.20 gm. of pepsin in N/10 hydrochloric acid and 2 cc. of tricresol. The mixture was brought to a volume of 400 cc. with N/10 hydrochloric acid and incubated in stoppered flasks at 37 to 38°C. At definite intervals 3 cc. samples of the mixture were removed, filtered and treated with 1 volume of sodium hydroxide to inhibit further peptic digestion. These samples were then analyzed for amino nitrogen by the micro method of Van Slyke ('13).

After 71 hours of digestion with pepsin-hydrochloric acid, 300 cc. of digest were withdrawn, made faintly alkaline with solid sodium bicarbonate and treated with 0.4 gm. of trypsin. These solutions were again incubated at 37 to 38°C. Samples were withdrawn at definite intervals, treated with one-third their volume of glacial acetic acid to inhibit further proteolysis and analyzed for amino nitrogen.

After 150 hours of digestion with trypsin, 75 cc. of the tryptic digest were treated with 25 cc. of erepsin solution and again incubated. Samples from this were analyzed as in the case of the tryptic digest.

A summary of the results is given in table 2. In this summary all the figures given have been corrected for the amino nitrogen liberated by the enzyme controls. The amino nitrogen present in the spinach leaf before digestion, namely, 2.93 mg. per gram of the dried leaf, has also been subtracted from each result.

In order to obtain data on the digestibility of casein, this protein was treated in the manner already described for the spinach leaf. For purposes of comparison the results of the digestion of 5 gm. casein by this method are included in table 2.

Other experiments conducted to test the validity of the digestion technic included a study of the effect of the activity of diastase on the proteolytic digestion of spinach. Carman, Smith, Havens and Murlin ('29) have intimated that proteolytic digestion, when conducted in the presence of diastase,

will be carried further than such a digestion in the absence of diastatic activity. That this was not true in terms of amino nitrogen was shown by duplicating the above described digestions of dried spinach and adding diastase to one series along with the other proteolytic enzymes. There was no increase in the amount of amino nitrogen liberated due to the presence of diastase.

TABLE 2

Summary table showing per cent of total amino nitrogen[1] liberated from spinach and casein by pepsin, trypsin and erepsin

PEPSIN			TRYPSIN			EREPSIN		
	Spinach	Casein		Spinach	Casein		Spinach	Casein
hours	%	%	hours	%	%	hours	%	%
1	8.3	8.6						
18.5	12.3	13.1						
27	13.1	13.3						
50	13.9	14.1						
71	16.3	14.9						
73			2	25.1	38.8			
97			26	34.7	58.2			
118			47	37.1	61.0			
143			72	37.9	63.1			
169			88	37.9	63.4			
191	20.3	17.4						
195			124	38.7	63.4			
221			150	38.7	63.4			
221.5						0.5	42.0	66.0
241						20	56.4	87.8
263						42	62.0	89.7
282						61	63.6	89.9
298						87	63.6	89.9
336			265	38.7	63.4			

[1] Twenty-five and 98.0 mg. total NH_2N per gram of dried spinach and casein, respectively.

Discussion and a suggested method for determining available nitrogen

A comparison of the amino nitrogen liberated from the uncooked green leaf with that of casein (fig. 1) seems to indicate that the protein of spinach is not as easily digested as

casein since only about 40% of the amino nitrogen of the spinach was liberated as compared with 62% from the casein by pepsin plus trypsin. However, examination of the work of Waterman and Jones ('21) shows that there is a marked increase in the digestibility of some proteins after cooking. Therefore, any future work on this problem would have to include a study of the digestibility of the cooked vegetable since this is the form in which the food is consumed by man.

On the basis of the above results and of those reported by Frankel ('16) and Waterman and Jones ('21) one is strongly tempted to make the statement that vegetable proteins are not

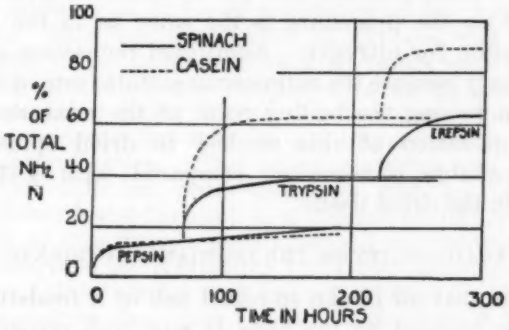

Figure 1

digested as well as casein in the alimentary tract. However, our knowledge of the mechanisms of digestion and absorption in vivo is still too limited to warrant such a conclusion. The recent studies of London ('34) showing that polypeptides are absorbed from the intestine suggest that any attempt to break down a protein completely to amino acids in order to simulate what happens in the alimentary tract, may not be the appropriate objective.

A more practical procedure, perhaps, would be to estimate with greater accuracy the available nitrogen in a food. By eliminating the fat-soluble nitrogen and the nitrate, amide and ammonia nitrogen before determining protein and amino

acid nitrogen, a more useful figure can be obtained. The following method was devised for that purpose:

Extract 2 gm. of the dried finely comminuted substance three times with 50 cc. portions of a hot alcohol-ether mixture containing 80% alcohol and 20% ether. Transfer the residue from this extraction to a Kjeldahl flask, and mix with 50 cc. water, 2 gm. of ferrous sulphate and 5 cc. of H_2SO_4 (1 + 1). After the reaction has ceased, boil the mixture slowly for about 5 minutes and cool. Add water, to make a volume of about 100 cc., and about 5 gm. of magnesium oxide. Boil the mixture until almost dry, cool and carefully add 25 cc. of concentrated sulfuric acid and 1 drop selenium oxide. From this point on the procedure is the same as in the Kjeldahl determination for nitrogen. Additional potassium sulfate is not necessary because the magnesium sulfate formed is equally efficient in raising the boiling point of the mixture.

The application of this method to dried spinach gave 3.85% available nitrogen as compared with 4.41% total nitrogen in the dried tissue.

AVAILABILITY OF THE INORGANIC NUTRIENTS

The fact that all of the so-called ash of a foodstuff is not completely utilized by the body is now well recognized. A considerable portion of the inorganic compounds of the normal diet fails to dissolve during its passage through the alimentary tract and is therefore not absorbed. Because of their obvious importance, calcium, phosphorus and iron compounds have received most of the attention devoted to the availability of the inorganic substances.

No attempt will be made to review the extensive literature on the availability of the inorganic constituents of the diet. Typical papers in the field are those of Fincke and Sherman ('35), Sherman, Elvehjem and Hart ('34) and Blatherwick and Long ('22) on the utilization of calcium, iron and phosphorus, respectively.

Experimental

In the hope that application of in vitro digestion to the study of the availability of inorganic salts might prove informative, several experiments were conducted in which dried spinach was digested with enzymes. The amount of calcium, phosphorus, iron (and in a few cases the amount of chlorine) made soluble by this treatment was determined. The results of these experiments were compared with those yielded by chemical analyses for total calcium, phosphorus and iron.

Chemical analyses for total calcium, phosphorus and iron. About 5 gm. of dried spinach were weighed into a platinum dish and mixed with 15 cc. of a solution of 5% pure sodium carbonate. This was evaporated to dryness and carefully charred over a free flame until no more fumes were given off. The ashing procedure was completed in an electric furnace at a temperature below 550°C. for 8 hours. Particles of carbon may remain but they do not interfere with the subsequent determination. The ashed material was then extracted, first with hot water and then with hot nitric acid (1 + 4), filtered and brought to a volume of 100 cc.

Aliquots of this filtrate were analyzed for chloride by the method described by Whitehorn ('21), for calcium by Clark and Collip ('25) technics and for phosphorus by the gravimetric procedure described in the "Official and Tentative Methods of Analysis" (A. O. A. C., '30, p. 15). In order to determine iron, 25 cc. of the filtrate were mixed with 5 cc. of concentrated hydrochloric acid and evaporated to dryness on a hot-plate to drive off the nitrate. The dried material was dissolved in a solution of weak sulphuric acid (1 + 25), heated almost to boiling, and the copper precipitated by saturating the solution with hydrogen sulphide. The cooled solution was filtered and the iron in the filtrate oxidized to the ferric state by adding 1 cc. of bromine water. The excess bromine was removed by boiling the solution for 3 minutes. The removal of bromine was greatly accelerated by having a small glass bead present in the boiling solution. This solution was then cooled, saturated with carbon dioxide, mixed with 3 cc. of a 30% solution of potassium thiocyanate and titrated with approximately N/1000 titanium sulphate in an atmosphere of carbon dioxide.

The amounts in per cent of calcium, phosphorus, iron and chloride in the dried spinach were, respectively, 1.016 (± 0.006), 0.886 (± 0.002), 0.0541 (±0.0002) and 1.267 (± 0.004). These figures are averages of three determinations.

Application of in vitro digestions to determine the availability of the inorganic constituents of spinach. The procedure applied here was the same as that which was used to determine loss of total solids, above described in the discussion of protein digestion.

TABLE 3

Availability of the inorganic constituents of spinach as estimated by in vitro digestion experiments

DIGESTIVE AGENT	AMOUNT EXTRACTED			
	Chloride	Calcium	Phosphorus	Iron
	%	%	%	%
Water	99.5	8.6	96.6	9.0
N/10 HCl		29.1	80.6	18.6
0.5% Na_2CO_3	99.3	12.5	90.4	25.0
N/10 HCl followed by 0.5% Na_2CO_3		30.0	89.0	24.9
N/10 HCl plus 20 mg. pepsin		30.5	82.5	40.0
0.5% Na_2CO_3 plus 50 mg. trypsin	100.0	12.5	90.0	24.0
Pepsin followed by trypsin		30.2	89.9	40.6

The filtrates were mixed with 0.5 gm. of sodium carbonate and evaporated to dryness. The dry material was carefully charred, placed in a cold oven which was then brought to a temperature of 550°C. and kept there for about 8 hours. The resulting ash was extracted first with hot water and then with hot dilute nitric acid, filtered and brought to a volume of 50 cc. Aliquots of this were used to determine calcium by the method of Clark and Collip ('25). In those digestions in which hydrochloric acid had not been used, chlorine was also determined. A portion of the filtrate was freed of nitrates and analyzed for phosphorus by the method of Fiske and Subbarow ('25), and for iron by means of the titanium titration described above.

The results of these analyses, expressed in terms of percentage of total material extracted, are given in table 3.

DISCUSSION

An examination of the results in table 3 brings out some interesting correlations between the availabilities of calcium, phosphorus and iron in vegetables as reported in the literature, and their solubilities after in vitro digestion.

The chloride of the leaf is, of course, completely soluble. In fact, some methods of feed analysis determine the total chloride present by extracting the material to be analyzed with hot water (A. O. A. C., '30).

That the phosphorus in vegetables is readily available has been championed by Blatherwick and Long ('22) and others. It is thereore not surprising to observe that nearly all of the phosphorus of spinach is extracted by water alone. Only about 30% of the total calcium present could be made soluble after treatment with N/10 hydrochloric acid, and there was no significant increase in calcium solubility when the leaf was digested with pepsin. This may serve to explain the observation of Sherman and Hawley ('22) that the calcium of vegetables is not as available as the calcium of milk. More recently Fincke and Sherman ('35) have demonstrated by means of biological tests that the calcium of spinach is poorly utilized.

Sherman, Elvehjem and Hart's ('34) method of determining iron availability by extracting the material with 10% acetic acid and then estimating the amount of iron in solution by Hill's bipyridine method ('30) gave results indicating that only about 20% of the iron in spinach was available. The data in table 3, showing that more iron was rendered soluble by hydrochloric acid plus pepsin than by hydrochloric acid alone, may be taken as indicating that more than 20% of the iron in spinach is available.

SUMMARY

A study of the known in vitro digestion technics failed to produce a practical method that might be used to determine the utilizable nitrogen in the green leaf. The results obtained, however, suggested that a closer approximation to the available nitrogen could be obtained by eliminating the fat soluble

nitrogen, by means of extraction with an alcohol-ether mixture, and by boiling off the nitrate, ammonia and amide nitrogen from the substance to be analyzed before determining protein and amino acid nitrogen. On this basis a simple procedure for estimating the available nitrogen present has been devised.

An investigation of the digestion of spinach by successive treatment with pepsin, trypsin and erepsin was made. The results obtained when the entire leaf was digested are similar to those obtained with pure proteins. This indicates that with certain materials, at least, it is possible to perform exact proteolytic digestions without necessarily isolating the protein.

The application of in vitro digestions to determine the availabilities of the calcium, iron, chloride and phosphorus in spinach confirmed the view advanced in the literature that the calcium and iron in spinach is only partly available. Enzymatic digestion brought about 30% of the calcium and 40% of the iron into solution. Chloride and phosphorus were readily dissolved in water alone.

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THE AVAILABILITY OF THE CARBOHYDRATES AND FATS OF THE GREEN LEAF TOGETHER WITH SOME OBSERVATIONS ON CRUDE FIBER

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CARBOHYDRATE

The need for more precise knowledge of the carbohydrate content of food has been apparent in recent years because of the necessity of preparing accurate food tables for diabetic diets. In 1920 Olmstead published a method for determining the availability of carbohydrate in vegetables by digesting with taka-diastase and then following the enzyme digestion with an acid hydrolysis in order to convert the maltose to glucose. This procedure was used on vegetables by O'Reilly and McCabe ('21) and Bell, Long and Hill ('25). Modifications of this method were introduced by Morgan, Strauch and Blume ('29) in their work on the almond carbohydrate and by Adolph and Kao ('34) who studied the carbohydrate of the soy bean.

Another taka-diastase method that has been described is that of Thomas ('24) who used this enzyme preparation without a secondary hydrolysis. He claimed that under the conditions of his method the ratio of glucose to maltose formed

[1] The data in this paper were taken from a dissertation presented by M. K. Horwitt in partial fulfillment of the requirements for the degree of doctor of philosophy, Yale University, 1935. Lack of space prevents the presentation of more detailed data than are reported here. Additional details are in the dissertation deposited in the Yale University library.

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was approximately constant, and that if he regarded the ratio as 2.0, the error was small providing the total reduction was determined by the picric acid reduction method. Widdowson ('31) used a taka-diastase digestion of starch and estimated the maltose and glucose formed by a modification of the Hagedorn-Jensen ('23) method.

The most extensive examination to date of the available carbohydrate in foods is that made by McCance and Laurence ('29). The total available carbohydrate of a large number of fruits and vegetables was estimated by hydrolyzing each food with hydrochloric acid and then determining the total reducing sugar. A correction was made for any pentosans which might have been hydrolyzed. More recently Widdowson and McCance ('35) reported a study on the available carbohydrate of a few fruits in which the glucose, fructose, sucrose and starch were determined individually. The sum total of these was called the available carbohydrate. In their paper they admitted that the hydrochloric acid hydrolysis used by McCance and Laurence ('29) destroyed 50% of the fructose.

Every carbohydrate found in nature may be found as a component of our food. Street and Bailey's ('15) investigation of the soy bean is an excellent illustration of the amount of work which has to be done in order to estimate the carbohydrate content of a food. These investigators determined pentosans, galactans, organic acids, invert sugar, sucrose, raffinose, starch, cellulose, dextrin and waxes. A similar study was performed on the navy bean by Petersen and Churchill ('21).

The digestibility of these various components has been a major problem in nutrition for many years. McCance has presented an excellent summary of the food value of vegetable carbohydrates (McCance and Laurence, '29).

Progress in the chemistry of the carbohydrates of foods has advanced slowly not for lack of efforts in this field, but rather because of the absence of relatively simple methods. Many investigators have carefully fractionated the carbohydrate portion of a few foods into the various components,

but each such attempt has constituted a complex problem in itself and the technic used has been too involved to find favor in the routine analyses of foods.

Since no one of the existing methods was entirely satisfactory, an attempt was made to devise a simple enzymatic procedure which, nevertheless, would not sacrifice accuracy for the sake of simplicity.

Choice of enzymes. An investigation of the literature showed that both diastase and taka-diastase had been used rather indiscriminately in attempts to determine digestible carbohydrates. In 1925, R. Kuhn published an investigation on the nature of the amylases in pancreatic juice, in malt diastase and in taka-diastase. He showed that both pancreatic amylase and the amylase in taka-diastase hydrolyzed starch to alpha maltose, whereas malt amylase hydrolyzed starch to beta maltose. From this one can conclude that the type of hydrolysis that taka-diastase catalyzes is more like that which occurs in the animal organism than that which is brought about by malt diastase.

The majority of taka-diastase preparations appearing on the market contain large amounts of lactose which is used as a solid diluent to bring the enzyme mixture to a standard strength. For exact work it is better to obtain a preparation that is free from extraneous reducing sugars. After trial and error, a particular commercial preparation of taka-diastase[3] was chosen, which will liquefy 2700 times its weight of starch in 10 minutes. The samples of this enzyme preparation which were used in the following experiments had very little, if any, reducing sugar present.

Choice of method for determining 'glucose.' Six different sugar methods were thoroughly tested before any one was chosen. Two of these were colorimetric, namely, Dastur and Samant's ('33) modification of the Folin-Wu procedure and Benedict's ('31) colorimetric copper method. Dastur and Samant added salts to each standard glucose solution so that the concentration of the ions in the standard was equal to

[3] Clarase 900% obtained from the Takamine Laboratories, Inc., Clifton, N. J.

that in the unknown. The theory behind this modification is excellent, but in actual practice this method proves too complex. Benedict's method uses a reagent devised to be only faintly alkaline in reaction. This modification works very well with blood filtrates but, when applied to the strongly buffered lead-clarified filtrates, the alkali is probably not strong enough. The same criticism can probably be made of the methods of Somogyi ('26) and of Hagedorn and Jensen (Hanes, '29), if these methods are to be applied to green leaves. Benedict's ('11) copper titration method was also used. The chief objection to this titration was the difficulty in obtaining good end points with dilute sugar solutions.

The method finally chosen was the Eynon and Lane ('23) titration. The methylene-blue end point is good; the method found to yield satisfactory reproducible results. Furthermore, the alkali used is sodium hydroxide, so that the buffering activity of the sugar filtrate has less influence than in the various methods that have been adapted to blood analyses. Eynon and Lane ('31) have suggested a modification of their method in which, by adding known amounts of sugar to the Fehling's solution before titrating, they are able to determine as little as 0.01% of sugar with desired exactness. Their method has been adopted as 'tentatively official' by the Association of Official Agricultural Chemists (A. O. A. C., '30).

Recovery experiments using this technic gave results within an error of 0.4%.

The active maltase in the taka-diastase preparation. That taka-diastase will convert starch to a mixture of maltose and glucose has been an accepted fact. According to the method most commonly used in sugar analyses, the hydrolysis of maltose to glucose is completed by heating with 2.5% hydrochloric acid at 100°C. for 2½ hours. In order to check this technic several determinations were made in which the reducing power of the clarified digest was estimated before and after the hydrochloric acid hydrolysis. It was found that in every case the acid hydrolyzed solution gave results that were 3 to 4% less than the digest which had not been hydrolyzed and which supposedly contained a large percentage of maltose.

A review of the literature offered two possible explanations of this phenomenon. In the first place, Davis and Daish ('13) had shown that it is impossible to convert quantitatively maltose to glucose by acid hydrolysis; the effects of variation of temperature, strength of acid and maltose concentrations were studied, but nevertheless, a destruction of a considerable amount of sugar occurred in every trial. Secondly, on comparing the amount of starch present in our digests with the amounts employed by other investigators, it was noticed that we were digesting relatively less polysaccharide than that hydrolyzed in other comparable studies. This suggested that diminution of the concentration of the substrate increases the

TABLE 1

Hydrolysis of maltose to glucose by Taka-diastase. (Maltose in amount stated, 0.025 gm. taka-diastase 15 cc. of buffer at pH 4.6 made to 100 cc. and incubated at 38°C. for 48 hours with 1 cc. of toluene)

MALTOSE HYDRATE	REDUCING POWER OF DIGEST EXPRESSED AS GLUCOSE	'GLUCOSE'/MALTOSE
gm.	*gm.*	%
1.980	1.690	85.3
0.990	0.891	90.0
0.495	0.473	95.5
0.247	0.246	99.6
0.1485	0.1480	99.7
0.0990	0.0994	100.4

extent of hydrolysis of maltose by maltase. When maltase is allowed to act on a concentrated solution of glucose, maltose is formed; when the concentration of the glucose is decreased, the amount of maltose thus synthesized is decreased.

A search for studies of the effect of taka-diastase on dilute starch solutions revealed the note by A. C. Hill ('02) in which is mentioned the fact that commercial taka-diastase completely hydrolyzes dilute starch solutions to glucose.

In order to test this maltase activity, solutions containing different concentrations of purified maltose hydrate were digested with 25 mg. of the special taka-diastase preparation for 48 hours as described in table 1.

At the end of the digestion the solutions were carefully analyzed for reducing power by the Lane-Eynon titration. The results expressed as glucose are given in table 1. It is evident that the smaller the concentration of maltose used, the greater was the extent of hydrolysis. Maximum hydrolysis is obtained when the concentration of maltose hydrate is below 0.250 gm. per 100 cc. The fact that Davis and Daish ('14) reported a yield of about 80% maltose and 20% glucose after digesting 2 gm. of starch with taka-diastase gives these data added significance.

The results of this experiment explain why no additional glucose was obtained after treating the leaf digest with acid. Little maltose, if any, was present; it had apparently all been converted to glucose.

This information should greatly simplify any future analysis of starch or starch product.

Recovery experiments on three 220 mg. samples of thoroughly dried starch, which were boiled for 15 minutes in 50 cc. of water, cooled, and digested with 25 mg. of the taka-diastase in a total of 100 cc. at 38°C. for 48 hours, averaged 99.6 ($\pm$ 0.8)% recovery. The taka-diastase was added in two equal portions at 24-hour intervals. One-half cubic centimeters of toluene was used as an antiseptic and the pH was adjusted to 4.6 with acetic acid.

Modification of method to enable a correction for pentoses. That free pentoses are present in the leaf in small amounts has been shown by Davis and Sawyer ('14) who fermented the maltose and glucose present with yeast and interpreted the residual reducing material as pentose. Any digestion with taka-diastase of a material containing hemicellulose will bring about hydrolysis of some of this carbohydrate to pentose because of the presence of a cytase in taka-diastase. Since pentose is a reducing sugar a correction must be made. Fortunately, pentose is not fermented by yeast and the available monosaccharides found in vegetable foods, glucose and fructose, are completely destroyed by yeast zymase. Whatever sugar remains after digestion of a mixture by yeast may

properly be considered as non-available. Therefore, a part of each clarified digest is treated with fresh, thoroughly washed, starch-free yeast in order to estimate the non-fermentable sugars. The total sugar obtained after digestion minus the non-fermentable sugars is taken as the available carbohydrate in the food.

Sucrose hydrolysis by invertase. The disaccharide, sucrose, is one of the constituents of the plant cell and any method which determines available carbohydrate must take account of this sugar. The enzyme, invertase, which quantitatively converts sucrose to fructose and glucose, has in recent years been studied more thoroughly than any other carbohydrase. Furthermore, active preparations of invertase are now available on the market.[4] The inversion of sucrose is adequately treated in the Official and Tentative Methods of Analysis (A. O. A. C., '30) and need not be discussed further.

Amounts of enzyme and duration of digestion. Fortunately for the method to be proposed, the reducing power of invert sugar and glucose are so nearly alike when determined by the Lane-Eynon methylene-blue titration that for practical purposes the difference can be ignored. The amount of sucrose present in the average vegetable is small compared to the quantity of glucose-yielding carbohydrates and the error is therefore well within the limits of the digestion error if all the sugar is expressed as glucose.

Two other variables, the amount of enzyme to be used and the duration of the digestion, had to be studied before the method could be considered adequate. In the preliminary experiments, 25 mg. of the special taka-diastase had been considered sufficient to complete the hydrolysis of starch in 40 hours; later 50 mg. were used in order to increase the margin of safety. In an effort to learn whether there would be any advantage in adding the 50 mg. in two or three lots, 24 hours apart, instead of adding it all at once, all three of these procedures were tested. The results of these experiments are given in table 2. A perusal of the figures in this

[4] Supplied by Wallerstein Laboratories, 171 Madison Ave., New York, N. Y.

table shows that it is best to add the special taka-diastase in two lots, 24 hours apart, and that there is no advantage in adding the enzyme preparation in three lots since the maximum availability is obtained after 50 hours. In actual practice it is not advisable to digest longer than 44 hours since Davis and Daish ('14) have shown that a loss of sugar may occur if starch is digested by taka-diastase for longer than 44 hours.

TABLE 2

Determination of available carbohydrate in dried spinach

		AMOUNT OF REDUCING MATERIAL RELEASED BY TAKA-DIASTASE AND INVERTASE	AMOUNT NOT FERMENTED BY YEAST	AVAILABILITY EXPRESSED AS GLUCOSE
	hours	%	%	%
(A)	16	10.18	2.24	7.94
Adding all of enzyme in one	32	10.18	2.24	7.94
lot at the start	45	10.58	2.34	8.24
	55	10.18	2.14	8.04
(B)	40	11.49	3.10	8.39
Adding taka-diastase in two	50	11.61	3.21	8.40
lots of 0.025 gm. each 24	70	11.55	3.21	8.34
hours apart	70	11.64	3.21	8.34
(C)				
Adding taka-diastase in three	70	11.75	3.32	8.43
lots of 17 mg. each, 20	70	11.73	3.32	8.41
hours apart				

The method in detail. An amount of dried, thoroughly powdered material (3 gm. spinach) containing less than 0.250 gm. of available carbohydrate is mixed with approximately 180 cc. of water and heated at 100°C. for 1 hour to gelatinize the starch. This is cooled to 40°C. and acidified with 5% acetic acid (about 12 drops) to pH 4.6. Twenty-five milligrams of the special taka-diastase and 1 cc. of toluene are added to the mixture which is then incubated at 38°C. At the end of 24 hours, an additional 25 mg. of taka-diastase together with 5 cc. of a solution of invertase 'free from melibiase' are added to the mixture. After 20 more hours in the incubator, 5 cc. of a saturated solution of neutral lead acetate are added to the digest which is then cooled to room temperature and diluted to a volume of 200 cc.

The mixture is filtered and a drop of methyl red added. Dry disodium phosphate is added, a little at a time, until the methyl red has lost its red color. Dry sodium carbonate is added until mixture is brought to pH 7,[5] after which it is filtered. This filtrate is divided into two parts; the first is analyzed by the Lane-Eynon titration for total reducing sugars, the second is fermented with yeast to give the non-fermentable sugars. The procedures are described below.

Seventy-five cubic centimeters of the clarified filtrate are treated with about 3 gm. of starch-free brewers' yeast which has been thoroughly washed by centrifuging three times with distilled water. This mixture is allowed to stand for ½ hour at room temperature after which time it is heated to 100°C., cooled, made to 100 cc., filtered and analyzed for reducing sugar by the Lane-Eynon titration. The calculated concentration of the non-fermentable sugars is subtracted from the total reducing sugar to give the available carbohydrate in the sample.

CRUDE FIBER

According to the Weende method (Henneberg, 1864) crude fiber is determined by boiling the dried food first with dilute acids and then with dilute alkali under strictly defined conditions of concentration and time, and then washing the undissolved residue with alcohol and ether. Henneberg was fully aware of the deficiencies of this method but claimed that its adaption would give an approximate determination of the cell residue. It is still used to determine crude fiber (A. O. A. C., '30).

In 1933, Remy described a biological method in which he used enzymes to separate cellulose, lignin and hemicellulose from starch, protein and fat. Remy compared the total of the cellulose, lignin and hemicellulose obtained as residues in enzymatic digestion with values obtained by the Weende method and concluded that about 50% of the indigestible residue is made soluble during the hydrolysis with acid and alkali. This new technic was a decided improvement over anything which had been suggested before.

[5] Nitrazine-yellow paper is a useful indicator in this range. It was described by Wenker ('34).

Williams and Olmstead ('35) recently published a modification of Remy's technic in which they separated the components of the indigestible residue into three fractions. That part not digested by enzymes which is not hydrolyzed by strong sulphuric acid is called lignin. The fraction made soluble by the sulphuric acid is analyzed for total reducing power. That part of the latter which is not fermentable is calculated as hemicellulose, inasmuch as hemicelluloses are converted to pentoses by acids. The remaining reducing power is calculated and interpreted as cellulose since cellulose is hydrolyzed to glucose by acids.

A discussion of other methods, intended as improvements on the old Weende technic, is given in the paper by Williams and Olmstead ('35).

A comparison of 'crude fiber' results obtained by enzymatic methods with those obtained with the so-called official method. The enzymatic digestion procedure used was similar to that described by Remy ('33) except that trypsin[6] was substituted for pancreatin and the special taka-diastase described above was used instead of the diastase used by Remy.

Three grams of dried spinach were incubated with 0.5 gm. of pepsin in 500 cc. of N/10 hydrochloric acid for 48 hours. The mixture was then neutralized to pH 7 with sodium hydroxide, brought to pH 4.5 with hydrochloric acid and treated with 0.1 gm. of taka-diastase for 48 hours. At the end of this time the digest was filtered, the residue returned to the digestion flask and treated with 500 cc. of a faintly alkaline solution containing an extract of 0.5 gm. of trypsin. This was allowed to incubate for 4 days, toluene being added daily. The residue from this digest was washed with water until the filtrate no longer gave a test for chloride, then with alcohol and ether, and dried at 110°C. to constant weight.

The data in table 3 show that the enzymatic treatment yields residues that are more than three times as large as the successive treatment with acid and alkali characteristic of the conventional crude fiber method. This is not surprising since it has long been known that much of the indigestible

[6] Obtained from Fairchild Bros. & Foster, New York, N. Y.

carbohydrate is dissolved by acid hydrolysis and therefore would not be in the residue. These data constitute further examples of the deficiencies of the accepted technics of food analysis.

THE ETHER-SOLUBLE FRACTION

Any investigation into the methods of food analysis would be incomplete if some attention were not given to the much neglected ether-soluble fraction of the food. The material commonly extracted from vegetable products and called 'crude fat,' 'ether extract,' or simply 'fat' is a mixture of several substances. One obtains not only true fats, that is, glycerides of fatty acids, but also lecithins, waxes, alkaloids,

TABLE 3

Comparison of biochemical method of obtaining crude fiber with the method of A. O. A. C. ('30)

	DRY SPINACH	CRUDE FIBER	CRUDE FIBER
	gm.	*gm.*	%
Method of A. O. A. C.	2.000	0.1672	8.36
	2.000	0.1701	8.50
	2.000	0.1690	8.45
Enzymatic method	3.000	0.8412	28.0
	3.000	0.8400	28.0

sterols, chlorophyll, xanthophyll and carotene. The method in which the ether extract is designated as the fat of a food dates back to early investigations of the Weende Experiment Station by Henneberg and has not been changed to any great extent since.

Digestibility of components of the 'ether extract.' The investigations of Langworthy and Holmes ('15, '17 a, '17 b) and Holmes ('18 a, '18 b, '19) on the availability of many different fats definitely showed that practically all true fats, animal or vegetable, are digested and assimilated by man. Less is known about the nutritive value of the other components of the ether extract. However, the results of recent experiments are enlightening to the extent that it is now possible to draw

tentative conclusions concerning the digestibility of these fat-soluble substances. Since lecithins are readily hydrolyzed by the digestive lipases (Sinclair, '34), these compounds can be considered completely available to the animal body.

Fischer and Hendschel ('33) studied the biological breakdown of chlorophyll in human beings. Experiments with a diet consisting mainly of spinach showed that chlorophyll was but slightly changed in the digestive tract. Magnesium and phytol are hydrolyzed from the chlorophyll molecule, but the main structure of the molecule, which is similar to that of hematin, is practically unaltered. The phylloerythrin formed may be partially absorbed but it is excreted in the bile.

That carotenoid compounds can be absorbed is proved by the phenomenon of carotinemia (Hess and Myers, '19). It is doubtful, however, whether these substances are available for energy metabolism because carotenes are very resistant to biological oxidation (Kuhn and Livada, '33). Salomon ('33) reported that the absorption bands of carotene and xanthophyll were found in fecal extracts even after 9 days of a diet devoid of these pigments. This may be taken to mean that those carotenoids which find their way into the blood stream are slowly excreted by way of the bile.

Recent studies on the metabolism of sterols indicate that the members of this class of compounds, though readily absorbed in many cases, are excreted in their original or only slightly changed state. On the basis of the work reported to date, one cannot consider the sterols as potential sources of energy.

It might be said, therefore, that the only parts of the ether-soluble fraction which are available as food are the fatty acids or compounds of the fatty acids. This should not be far from the truth, and for practical purposes of food analysis, a method which would separate the fatty acid compounds from the other components of the 'ether extract' might prove useful.

Experimental

Assuming that the total fatty acids present in the saponified alcohol-ether extract are an index of the availability of the fraction, the extract was saponified and an attempt made to isolate the fatty acids from the resulting mixture. The results were very discouraging because the solubilities in ether of chlorophyll and of fatty acids are so similar that it was difficult to isolate the fatty acids. For this reason experiments depending on the use of a saponification were discontinued for the time being.

In the hope that a successful method of determining the availability of the fat-soluble fraction might be developed if more were known about the lipases, an investigation with this type of enzyme was begun. The failure of all experiments of this type led to discontinuance of this line of investigation.

At this stage of the study, data on the solubilities of the chlorophylls were examined in the hope that one could separate these pigments from the fatty acids by the choice of suitable solvents. A sentence in a paper by Willstätter ('15, p. 334) proved particularly interesting. "When chlorophyll has reached a certain degree of purity, it is still easily soluble in alcohol containing petroleum ether but, surprisingly, no longer soluble in pure petroleum ether." This unexpected observation proved a great aid to Willstätter in his isolation of chlorophyll. It likewise proved helpful in the present study in separating the fatty acids from chlorophyll in a saponified alcohol-ether extract.

Determination of total fatty acids. The method finally developed is a modification of the Kumagawa and Suto ('08) saponification technic. The following procedure works satisfactorily when applied to the green leaf:

A 10 gm. quantity of the dried, powdered leaf (dried in an atmosphere of carbon dioxide to prevent oxidation of fats) is placed in a 200 cc. Erlenmeyer flask and boiled with 100 cc. of an alcohol-ether (4 + 1) mixture on a hot plate for 10 minutes. The blue-black supernatant fluid is decanted into a filter paper and the filtrate collected in a 250 cc. beaker. The residue is extracted for 5 minutes with 75 cc. of the boiling alcohol-ether mixture and twice again with 25 cc. portions.

The total filtrate, which now contains all the lipids and nearly all of the pigments, is evaporated to a volume of about 100 cc. To this solution 20 cc. of approximately 10 N potassium hydroxide are added. During saponification the mixture is heated on a water bath in an open beaker until the volume has been reduced to about 30 cc. At this point, 30 cc. of water are added and the saponification continued until the total time elapsed on the water bath is 1 hour.

The total saponified mixture, which should not have more than 20% alcohol, is acidified with hydrochloric acid. A large excess of hydrochloric acid should be avoided. A strongly acidified solution would cause the formation of chlorophyll products which are more soluble in petroleum ether (Willstätter, '15). The acidified mixture is transferred to a decantation flask and extracted with petroleum ether, twice with 50 cc. and twice with 30 cc. This extract is filtered and evaporated to a volume of 50 cc. It now contains all the fatty acid, the non-saponifiable lipids, some xanthophyll (which is not very soluble in petroleum ether), most of the carotene and traces of chlorophyll products. Most of the chlorophyll products have been removed; the remaining traces are separated out in the subsequent extractions.[7]

The petroleum ether extract which has been evaporated to 50 cc. is extracted with 50 cc. of N/10 potassium hydroxide in 50% alcohol. This in turn is twice extracted with 25 cc. of petroleum ether. To make certain that none of the fatty acids remain with the non-saponifiable substances the petroleum ether fractions are combined and extracted with 25 cc. of the alcoholic potassium hydroxide solution. The xanthophyll and most of the carotene have been left behind in the petroleum ether fraction with the non-saponifiable lipids and the alcoholic potassium hydroxide solution is now ready for the final extraction.

The combined potassium hydroxide fractions are acidified with hydrochloric acid, using a trace of phenolphthalein, and extracted with 50 cc., 25 cc. and 20 cc. portions of warm petroleum ether. The petroleum ether extract of the fatty acids is evaporated in a tared dish, dried at 100°C. for 30 minutes and weighed.

[7] The phytochlorins and phytorhodins when pure are insoluble in petroleum ether (Willstätter, '15); therefore, these compounds become less soluble, as one proceeds with the separation.

The total solids obtained by the above technic should contain practically all of the fatty acid and only minute traces of the pigments of the alcohol-ether extract.

The saponification technics suggested by Liebermann (1898) and Kumagawa and Suto ('08) involve a direct saponification of the tissues; they do not extract the total lipids before saponification. Several experiments with dried spinach showed that it was not possible to obtain results by saponifying vegetable tissue directly, probably because of the resistance offered by the cellulose structures of the plant. The alcohol-ether mixture is particularly efficient in reaching all parts of the cell and for this reason an extraction of the lipids with this solvent before saponification is to be preferred.

Application of method. Ten grams of powdered spinach (9.355 gm. of moisture-free material) were weighed into a 200 cc. Erlenmeyer flask, extracted with alcohol-ether and the total fatty acids present determined by the modified saponification technic described above. The average of three determinations showed that 0.210 ± 0.002 gm. of fatty acids were present in the spinach used or $2.23 \pm 0.02\%$. Since the ether-soluble fraction as determined by a standard Soxhlet method (Leach, '20) gives an average of 5.44%, it is evident that the fatty acids account for only 41% of the ether extract and the remaining 59% are unsaponifiable matter, pigments and some glycerol.

Speer, Wise and Hart ('29) have shown that a large percentage of the total fatty acids of the spinach leaf is present as free fatty acids. For this reason it is not a simple matter to estimate the amount of true fat (glyceride esters of fatty acids) present in a vegetable substance. However, in order to facilitate dietetic calculations, it would probably not be far from the truth if each gram of fatty acid obtained by the above method was evaluated as being equivalent to 9.5 calories of energy. The use of this tentative figure would be far more accurate than to continue the prevailing custom of assigning the value of 9.0 calories to each gram of the ether-extract.

DISCUSSION

All the methods mentioned above have been devised with the idea that they be applied not only to the green leaf but to foods and feeds in general. It is quite evident that if we are to determine the true caloric content of many of our foods we shall have to change our methods of analysis. Table 4 illustrates the differences obtained when the methods used in Atwater and Bryant's Bulletin no. 28 and the technic referred to in this paper and that of Horwitt, Cowgill and Mendel ('36) are applied to the same lot of spinach.

TABLE 4

Comparison of results of old and newer methods

	BULLETIN NO. 28	NEW
	%	%
Ash	19.3	
Crude fiber	8.4	28.0
Proteins[1]	27.6	24.0
Fat	5.5	2.2
Carbohydrate	49.2	8.4
Calories per 100 gm.	356.0	139.0

[1] N × 6.25.

It is appreciated that the green leaf is not fed to man because of its caloric content and that it affords an exaggerated example of the differences involved; but, nevertheless, one should endeavor to be more precise in one's statements of the food value of the vegetables in our diet.

SUMMARY

Carbohydrates. A study of the activity of taka-diastase showed that an active maltase capable of converting maltose to glucose was present. This conversion can be accomplished if the concentration of the maltose in the substrate is not more than 0.25 gm. in 100 cc. of solution. Use of this fact was made in devising a method for determining the available carbohydrate of a food.

Crude fiber. A comparison of the enzymatic procedure for determining the crude fiber of spinach with the so-called official method showed that the former gives results which are more than three times as large as those obtained by the present 'official method.'

Fats. A method for estimating the true fat content of the ether-soluble fraction of a food material is presented. The application of this method to spinach showed that more than 55% of the material obtained by ether extraction and sometimes called 'fat' is indigestible.

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THE EFFECT OF CYSTINE AND CASEIN SUPPLEMENTS UPON THE NUTRITIVE VALUE OF THE PROTEIN OF RAW AND HEATED SOY BEANS[1]

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Several investigators have analyzed the soy bean to determine the completeness of its protein. Osborne and Clapp ('07), Jones and Waterman ('21), Hamilton, Uyei, Baker and Grindley ('23), and Nollam ('15) showed that the protein of the soy bean contains in quantity all of the essential amino acids with the possible exception of cystine. Mitchell and Smuts ('32) and Shrewsbury and Bratzler ('33) claimed a quantitative deficiency of the amino acid, cystine, in the protein of the raw soy bean on the basis of biological experiments. However, Csonka and Jones ('34) reporting an analysis of a 10% sodium chloride extract of defatted soy bean meal—which represented 85 to 90% of the total nitrogen of the meal—obtained values for cystine which ranged from 0.287 to 0.491%. These values led them to question the existence of an actual deficiency of cystine in soy bean protein. They pointed out that different varieties of soy beans differed materially in their protein content. It was suggested that for practical feeding purposes it might be desirable to discriminate between different varieties in order to provide a satisfactory protein content of the ration.

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Hayward, Steenbock and Bohstedt ('36) found by metabolism trials that the protein of the soy bean was made more efficient by exposure to heat. With this exposure to heat there resulted only a slight increase in digestibility. Their results suggested that the application of heat to the raw soy bean caused an increase in the availability of certain fractions of the protein molecule. It seemed important that these findings should be submitted to more detailed analysis. In line with this we have now sought to determine if cystine or its equivalent represents that part of the protein fraction which is made more available by a process of heating. We have centered our attention primarily upon the effect of the process of heating which is used commercially in the extraction of the soy bean oil. In a supplementary way we have also carried out some experiments on the effect of casein when fed at different levels in supplementing the proteins of the raw soy bean. The primary objective of these experiments was to demonstrate if the beneficial effect of heat was exerted on the protein fraction only or whether the digestibility and availability of other constituents of soy beans were likewise improved.

EXPERIMENTAL

In our first series of experiments we fed a supplement of cystine with both raw and heated soy beans. The soy beans were of the Illini and Herman varieties. These were used because Csonka and Jones ('34) have reported that the Illini and Herman varieties differed considerably in their cystine content. We did not analyze our samples for cystine, but analyses for sulfur revealed that the Illini beans contained 0.344% sulfur and the Herman beans 0.382%. When extracted soy beans were fed, soy bean oil was added to restore the original fat content. The soy bean oil meal fed was the high temperature expeller meal prepared from Illini beans of the same sample as those fed in the control rations. The method of preparation of this expeller meal has already been published (Hayward, Steenbock, Bohstedt, '36).

In our second series of experiments casein was incorporated into the soy bean diet in the form of crude casein. Cystine was also added to check the completeness of the diet containing the raw soy beans and the intermediate level of casein.

All experimental diets were compounded so as to contain approximately 18% of protein. Actual analyses revealed a range in protein content from 17.6 to 18.3%. The basal ration in which the soy bean preparations were fed was composed of 45 parts cooked starch, 4 parts of salts number 40 (Steenbock and Nelson, '23), and 2 parts of cod liver oil. The amount of starch incorporated in the respective rations was adjusted inversely with the amount of soy bean products or other supplements required by analyses to supply the desired level of protein. l-Cystine was added as desired at a level of 0.3% of the ration. When yeast was added it was included at a 6% level.

Twenty-six lots of six male rats weighing from 50 to 60 gm. were used in the experiments. Each rat was quartered and fed separately. The food consumption of all rats was equalized from week to week. This was effected by reducing the intake of all rats to the intake of the rat which consumed the least, providing that this rat was not obviously abnormal (Steenbock, Black and Thomas, '29, '30). Each experiment was continued for 56 days. The results of the experiments are grouped together in table 1. They are expressed in terms of gain in body weight per gram of protein eaten (Osborne, Mendel and Ferry, '19). The average daily food consumption of all rats was 4.84 gm., which represented the amount consumed by the rats in lot 52 which received the raw soy bean diet.

It is to be noted that the ground raw soy beans of both the Illini and Herman varieties showed a low nutritive value as indicated by the grams of growth per gram of protein consumed. The addition of cystine or autoclaving the beans at 15 pounds pressure for 1 hour practically doubled the nutritive value of both varieties. Similarly, high temperature heating, as used in the expeller process of oil extraction, pro-

TABLE 1

Series 1: The effect of supplements of cystine, casein and yeast upon the nutritive value of the protein of raw and heated soy beans

LOT	DIET						GAIN, BODY WEIGHT		GROWTH PER GRAM OF PROTEIN EATEN
	Soy beans			Special supplements			Range	Average	
	Kind[2]	Process	Temperature						
							gm.	gm.	gm.
52	Ground beans A						8–26	18.6	0.38
54	Ground beans A					Yeast[1]	20–37	28.2	0.58
56	Ground beans A				Cystine		28–64	41.0	0.84
57	Ground beans A				Cystine	Yeast[1]	31–44	36.8	0.75
58	Oil meal	Expeller	150°C.			[1]	28–50	40.7	0.83
59	Oil meal	Expeller	150°C.			Yeast[1]	27–45	38.7	0.79
62	Oil meal	Expeller	150°C.		Cystine	[1]	40–53	46.5	0.95
63	Oil meal	Expeller	150°C.		Cystine	Yeast[1]	33–46	40.7	0.83
77	Ground beans A	Autoclaved	15 pounds 121°C. 1 hour				40–58	50.4	1.03
79	Ground beans A	Autoclaved	15 pounds 121°C. 1 hour		Cystine		43–59	49.7	1.02
70	Ground beans H						19–30	26.2	0.54
71	Ground beans H					Yeast[1]	28–35	30.2	0.62
72	Ground beans H				Cystine		37–51	44.5	0.91
73	Ground beans H				Cystine	Yeast[1]	37–44	40.8	0.84
76	Ground beans H	Autoclaved	15 pounds 121°C. 1 hour				46–62	55.0	1.13
78	Ground beans H	Autoclaved	15 pounds 121°C. 1 hour		Cystine		41–59	50.5	1.03
53	Ground beans A			Casein 2½%		[1]	29–36	31.1	0.64
55	Ground beans			Casein 2½%		Yeast[1]	27–39	32.7	0.67
64	Ground beans A			Casein 5%		[1]	24–44	36.8	0.75
65	Ground beans A			Casein 5%		Yeast[1]	30–42	37.2	0.76
66	Ground beans A			Casein 10%		[1]	40–51	46.2	0.95
67	Ground beans A			Casein 10%		Yeast[1]	42–53	47.8	0.98
68	Ground beans A			Casein 5%	Cystine	[1]	52–56	52.2	1.07
69	Ground beans A			Casein 5%	Cystine	Yeast[1]	38.51	44.0	0.90
74	Oil meal	Expeller	150°C.	Casein 2½%		[1]	35–53	45.3	0.93
75	Oil meal	Expeller	150°C.	Casein 2½%		Yeast[1]	37–51	43.8	0.90

[1] Soy bean oil added in minor amounts.

[2] The Illini variety of soy beans are designated with the letter 'A' and the Herman variety with the letter 'H.' The expeller soy bean oil meal was prepared from the Illini variety.

duced a decided increase in nutritive value. With the heated soy bean products, the addition of cystine did not produce unequivocal results; the nutritive value was apparently slightly improved with the expeller meal but was left unchanged in one of the autoclaved meals and somewhat reduced in the other. Evidently, the variations can be considered as due to experimental error. The addition of yeast to the raw soy bean diets caused a slight improvement in nutritive value, but with the diets containing the heated soy beans or the raw soy beans supplemented with cystine, yeast not only failed to improve the nutritive value of the protein but actually decreased it slightly. This was probably caused by the reduction in the amount of soy bean protein in the ration, which was made to keep the protein level constant. Apparently, yeast protein was not on a parity in nutritive value with the protein of the heated soy beans or the raw soy beans plus cystine. The fact that the addition of yeast to our heated soy bean rations did not produce any response in growth confirmed our surmisal that our rations were well supplied with the vitamins B.

The addition of casein to the raw soy bean diets caused an improvement in nutritive value. This increase was in proportion to the level of casein fed. When, in addition to 5 parts of casein, 0.3% of l-cystine was added, the nutritive value of the ration was again materially increased. In amount this was about the same as that produced with the raw soy beans upon the addition of 10 parts of casein. The incorporation of 2½% of casein in the heated soy bean diet failed to cause any material change.

DISCUSSION

The fact that cystine acts as an efficient supplement to the protein of the raw soy bean as also reported by Mitchell and Smuts ('32) and Shrewsbury and Bratzler ('33) might lead to the conclusion that soy bean protein is generally deficient in cystine. However, Csonka and Jones ('34) reported that soy bean oil meal contained from 0.287 to 0.491% of cystine when prepared, respectively, from the Illini and Herman

soy bean oil meal was prepared from the Illini variety.
[illegible] soy bean oil added in minor amounts.

varieties. These findings of Csonka and Jones and our sulfur values for the samples of soy bean of the Illini and Herman varieties used in our experiments seemed to us good reasons for suspecting that while cystine might be a limiting amino acid for the ray sow bean, this was not due to an actual deficiency but rather to a lack of availability of cystine or its equivalent. Of course, it is not excluded that cultural conditions have an effect upon the cystine content of soy beans and that other varieties than those used by us might even show greater departure from the values given. Conclusions in regard to this are not warranted until a general survey has been made. As our data are limited to the results obtained with samples of only two varieties, we can merely state that in some instances cystine or its biological equivalent is present in raw soy beans, at least in part in an unavailable form. Heating the soy bean apparently serves to correct or alleviate this deficiency.

The fact that the addition of yeast to the raw soy bean diets supplemented the proteins of soy beans to some extent is not surprising in view of the cystine content reported for yeast. Prunty ('33) analyzed various samples of yeast dried at 37°C. and found them to contain from 0.51 to 0.92%. Swift, Kahlenberg, Voris and Forbes ('34) found that yeast added at the rate of 1 gm. per rat per week to a diet purposely constructed to be deficient in cystine caused the rats to gain weight as rapidly as their mates which received a diet known to be satisfactory in cystine content. This additional gram of yeast per week was estimated to raise the cystine intake of the basal diet by about 12%. They attributed the increased growth to the cystine content of the yeast. We did not analyze our sample of yeast for cystine, but found that it contained 0.396% of sulfur.

Jackson and Block ('32) have reported that the amino acid methionine, like cystine, is capable of producing good growth in rats when added to a basal diet poor in cystine. Baernstein ('32) reported a value of 1.84% for methionine in glycinin—the isolated protein of the soy bean. He also reported that casein contained from 3.25 to 3.53% of methionine.

When we consider the cystine content of our soy bean diets in terms of the cystine values obtained by Csonka and Jones ('34) and the methionine value of Baernstein ('32), we obtain an appreciable value, viz., 0.25 to 0.35%. These differences in cystine equivalent values are offered as an explanation for the increased nutritive value obtained for the soy beans H, lot 76, over that for the soy beans A, lot 77, when both beans had received the same heat treatment, autoclaved 1 hour at 15 pounds pressure. These cystine equivalent values would be sufficient to explain why the diets containing the heated soy beans A and H failed to respond markedly to additions of cystine, if we assume that heating made this cystine equivalent fraction available for absorption and ultimate use in the animal's body. To support this supposition, other investigators (Rose, '31, '32; Jackson and Block, '32; and Swift, Kahlenberg, Voris and Forbes, '34) have found that the incorporation of 0.24 to 0.3% of l-cystine in a cystine deficient diet was sufficient to produce normal growth providing the diet was complete in other essentials.

It is known that casein ordinarily contains a variable but appreciable amount of cystine (Folin and Looney, '22; Jones and Gersdorf, '34) and methionine (Baernstein, '32). The results with different levels of casein then are not surprising in view of the cystine equivalent values for this protein and the fact that the protein of the raw soy bean can be supplemented by even small additions of available cystine as was shown in table 1.

The results of these experiments suggest that cystine or its equivalent may exist in the raw soy bean in a form which is at least partially unavailable to the rat. The application of heat, as for example, autoclaving the beans for 1 hour at 15 pounds pressure, or the use of the expeller method of oil extraction which involves heating the beans for 2½ minutes at a temperature of 140 to 150°C., caused the limiting factor to become available. Whether these differences lie in a failure of absorption or in a lack of availability after absorption remains to be demonstrated.

SUMMARY

In experiments with rats, raw soy beans of both the Illini and Herman varieties were found to contain an inefficient protein as measured by grams of growth per gram of protein eaten.

The additions of 0.3% l-cystine or the application of heat, such as autoclaving for 1 hour at 15 pounds pressure or the use of the expeller method of oil extraction, which entails heating the beans at a temperature of 140 to 150°C. for 2½ minutes, practically doubled the nutritive value of the protein. The addition of 0.3% of l-cystine to the heated soy bean diet failed to reveal an improvement which could be considered outside of possible experimental error.

Casein supplemented the protein of the raw soy bean and caused an increase in the nutritive value of the protein proportional to the amount added. The addition of 0.3% l-cystine resulted in an increase in nutritive value similar to that obtained when the high level of casein was added.

The fact that cystine supplemented the protein of the raw soy bean suggests that cystine or its equivalent may exist in the raw protein of the soy bean in a form which is not available to the animal. Since heating of the soy bean gave its protein a nutritive value practically equal to the nutritive value of the protein of the raw soy bean when supplemented with cystine, it appears that heating the soy bean caused the cystine fraction of the protein to become available.

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LOSSES OF VITAMIN C DURING THE COOKING OF PEAS[1]

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FOUR FIGURES

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Certain well-known investigators have reported that open-kettle cooking of vegetables is very destructive to vitamin C. Eddy, Kohman and Carlsson ('26) found 2 gm. daily of ungraded raw market peas, and 5 gm. but not 3 gm. of these ungraded peas cooked, protective against scurvy in guinea pigs. This gives a loss of approximately 75% of vitamin C during cooking. Bessey and King ('33) report, by dye titration, 0.16 mg. of vitamin C per milligram of fresh peas and 0.06 mg. per gram of cooked peas. McHenry and Graham ('35) found 0.14 mg. per gram of raw peas and 0.08 mg. per gram of cooked peas or a cooking loss of 43%. Fellers ('35) lists peas in a group of vegetables losing 40 to 80% of their vitamin C "during a short cooking period." He reports 3.6 gm. of cooked peas as the protective level. Recalculated, on a basis assuming 0.5 mg. of ascorbic acid per day are required to protect a guinea pig from scurvy, this is 0.14 mg. of vitamin C per gram of cooked peas. He gives no data on the raw pea content.

In the studies on peas published previously, no consideration has been given to the cooking water, perhaps partly

[1] Approved by the director of the New York State Agricultural Experiment Station for publication as Journal Paper no. 128, March 11, 1936.

because it is difficult to get animals to eat the large amount of cooking water produced. The new chemical method offers a very simple means of determining the amount of ascorbic acid that goes into the water. Our purpose was to determine not only the ascorbic acid retained in the peas, but also that retained in the cooking water. From the standpoint of human nutrition, the results of animal feeding experiments do not give as exact information as do the chemical determinations, inasmuch as human beings commonly eat food before it is cooled.

In this study it was possible to control the variety, maturity, and freshness of the peas, as well as the method of cooking. The main object was to measure the relative amounts of vitamin C in the peas and in the cooking water at the 'done' stage and at various intermediate stages, as well as losses during the standing of cooked peas. Every effort was made to control all of the factors in such a way as to cause minimum loss of vitamin C with the exception that the aim in cooking was to make the cooked vegetable look attractive and taste well, with the loss of nutrients a secondary consideration.

EXPERIMENTAL

1. Losses of vitamin C during the cooking of peas. Two varieties of peas, Thomas Laxton and Alderman, were grown on upland soil (Ontario clay loam at Geneva, New York). They were hand-picked when they reached the succulent stage, hand-shelled, cooked, samples extracted, and titrated in as short a time as possible.

The method of cooking was essentially that of Halliday and Noble ('33), with the exception that 525 gm. (six servings) of peas instead of 350 gm. were used with the 700 cc. water and ¾ teaspoonful salt. A 3-quart enamel pan with an inside diameter of 7 inches was used. The peas were dropped into the rapidly boiling water and the time counted from the time the water came back to the boil (this took 2 minutes). The tap water (pH approximately 7.5) was boiled several minutes before the peas were added.

An arbitrary stage of 'doneness' for each variety was determined in advance by several judges, using the common household methods of testing with a fork and 'biting.' The boiling time for the Thomas Laxton and the Alderman varieties was 14 and 13 minutes respectively. Samples of the peas and of the cooking water were removed at intervals from the beginning of cooking to the 'overdone' stage. Samples were also taken at the 'done' stage from normal cookings in which there had been no interference from previous sampling.

The method of extraction, of standardization of the dye, and titration were essentially those of Bessey and King ('33). Trichloracetic acid[2] was found to give a clearer extract of peas than acetic acid. Blanks were run with each dye used.

The samples of peas containing approximately 20 gm. were placed immediately into weighing bottles containing 25 cc. of 8% trichloracetic acid. The weighing bottles containing the acid had been previously placed in an ice-salt mixture so that when the hot peas were added to the chilled acid, the whole was brought to room temperature in a very short time.

Immediately after removal of each sample for vitamin C determinations, samples were removed for moisture determinations so that the vitamin content was calculated on both the wet and the dry weight.

The cooking water in 25 cc. amounts was pipetted into 25 cc. chilled trichloracetic acid in 50 cc. volumetric flasks.

There was little change of acidity, which might affect vitamin C stability, during the cooking. The total range of pH of the raw and cooked peas was from 6.30 to 6.88, indicating the presence of considerable buffer material in peas. The pH of the tap water used in cooking and of the cooking water at the 'done' stage was approximately 7.5 and 6.5 respectively.

For purposes of comparison, biological assays were made on the raw and the 'done' peas. The whole lot of one variety for animal feeding was picked and cooked on the same day.

[2] Mack, Tressler and Dearborn ('36) have shown that 8% trichloracetic acid prevents enzymic oxidation of ascorbic acid in fresh peas so that regeneration with H_2S is not necessary.

The 'done' peas were drained, cooled approximately 1½ minutes in shallow pans surrounded by ice and salt and then packed, sealed, frozen and kept in a container with dry ice.

The biological assay of the four samples of peas was based essentially upon the curative feeding test, in comparison with the response of animals receiving known amounts of the pure vitamin. The method was the same as that reported by Tressler, Mack and King ('36). Guinea pigs weighing about

TABLE 1

Vitamin C assay of cooked and raw peas

TEST FOOD	WEIGHT OF PEAS FED	VITAMIN LEVEL	NUMBER OF ANIMALS	AVERAGE WEIGHT AT BEGINNING OF TEST	AVERAGE CHANGE IN WEIGHT DURING TEST	SCURVY SCORE
	gm.	*mg. per day*[1]		*gm.*	*gm.*	
Thomas Laxton peas, raw	2.17	0.5	6	323	+ 34	3
Thomas Laxton peas, cooked	9.10	1.0	3	316	+ 55	0
Alderman peas, raw	2.08	0.5	5	323	+ 57	2
Alderman peas, cooked	3.33	0.5	5	322	+ 40	3
Standard solution[2]		1.0	6	310	+ 67	0
Standard solution		0.5	7	311	+ 41	2
Basal diet only		0.0	6	329	—113	19

[1] Quantity fed based upon values obtained from preliminary and concurrent indophenol titrations.

[2] The standard solution contained 1.0 and 0.5 mg. of ascorbic acid.

300 gm. at the time of purchase were fed a Sherman basal diet (Sherman, La Mer and Campbell, '22) (rolled oats, bran, butterfat, heated skimmilk powder, and salt), supplemented with cod liver oil and 4% yeast. A liberal allowance of crisp carrots or spinach was given during a preliminary period of 10 days, to eliminate any animals that appeared subnormal in growth rate or health. They were then kept on the basal diet without a vitamin C supplement for 11 days to deplete their tissue reserves, as indicated by a lessened or negative growth rate. The test peas were then given to matched groups in

TABLE 2

Vitamin C losses from peas and gain in the cooking water

LENGTH OF COOKING PERIOD IN MINUTES[1]	THOMAS LAXTON VARIETY			ALDERMAN VARIETY		
	Ascorbic acid			Ascorbic acid		
	Milligram per gram drained peas		Milligram per cubic centimeter cooking water	Milligram per gram drained peas		Milligram per cubic centimeter cooking water
	Wet weight	Dry weight		Wet weight	Dry weight	
0 (raw)	0.23	0.99	0.00	0.24	0.95	0.00
1	0.16	0.74	0.04	0.21	0.83	0.02
3	0.11	0.50	0.05	0.19	0.74	0.03
4	0.11	0.50	0.06	0.18	0.74	0.05
6	0.11	0.48	0.07	0.17	0.69	0.06
8	0.10	0.44	0.08	0.15	0.59	0.07
10	0.10	0.43	0.08	0.15	0.60	0.08
12	0.10	0.41	0.09	0.14	0.62	0.08
14	0.09	0.40	0.11	0.13	0.53	0.10
16[2]	0.08	0.38	0.11	0.12	0.52	0.12
18	...	...	0.13	...	...	0.14

[1] In all cases approximately 2 minutes were required to bring the water back to boiling after the peas were put into it, hence the peas cooked 1 minute had not been boiled, while those cooked 3 minutes had been boiled for 1 minute.

[2] Thomas Laxton peas were done in 16 minutes, the Alderman in 15 minutes.

TABLE 3

Vitamin C losses from cooked peas and gain in the cooking water. Samples not taken until peas were done

LENGTH OF COOKING PERIOD IN MINUTES[1]	CONDITION OF PEAS	THOMAS LAXTON VARIETY				ALDERMAN VARIETY			
		Ascorbic acid				Ascorbic acid			
		Milligram per gram drained peas		Milligram per cubic centimeter cooking water	Weight of cooking water	Milligram per gram drained peas		Milligram per cubic centimeter cooking water	Weight of cooking water
		Wet weight	Dry weight			Wet weight	Dry weight		
					gm.				gm.
0	Raw	0.23	1.01	0	700	0.25	0.96	0	700
15	Just done	...	...	...	...	0.15	0.57	0.12	440
16	Just done	0.11	0.44	0.13	430	...	...	...	
17	Overdone	...	...	...		0.14	0.54	0.14	
18	Overdone	0.11	0.52	0.14		...	...	...	
19	Overdone	...	...	...		0.13	0.51	0.16	
20	Overdone	0.12	0.53	0.18		...	...	...	
22	Overdone	0.12	0.54	...		0.14	0.52	0.19	
24	Overdone	...	...	0.26		...	...	...	
25	Overdone	...	...	...		0.13	0.54	0.20	
28	Overdone	...	...	0.39		...	...	...	

[1] In all cases approximately 2 minutes were required to bring the water back to boiling after the peas were put into it.

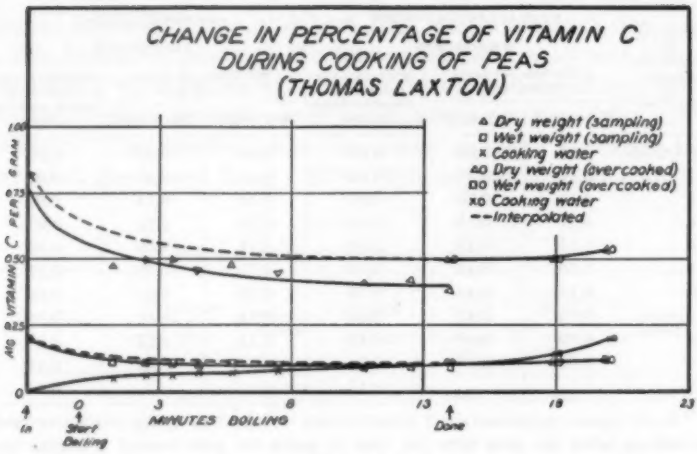


Figure 1

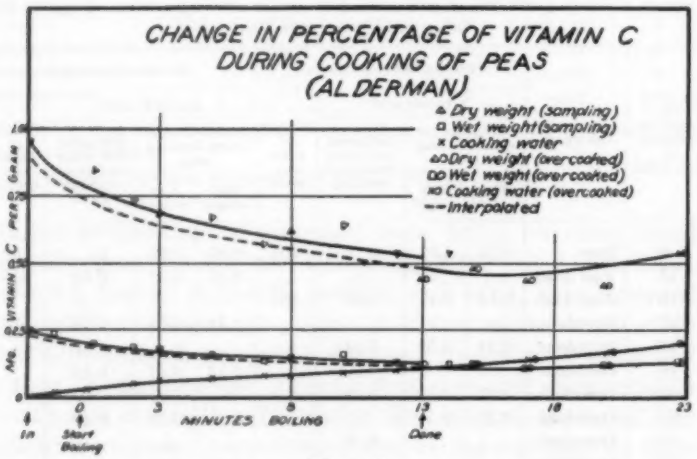
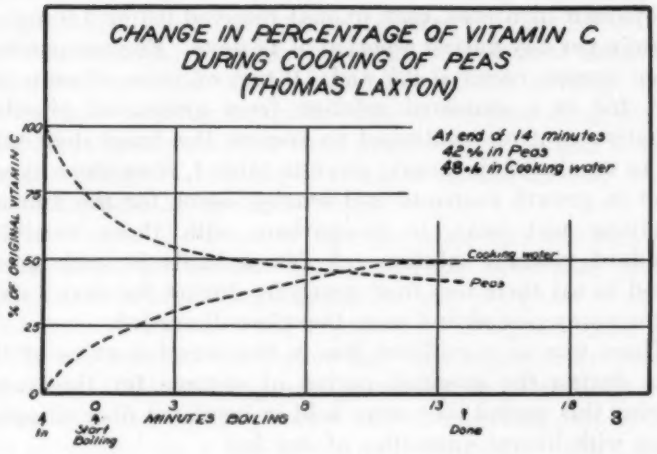
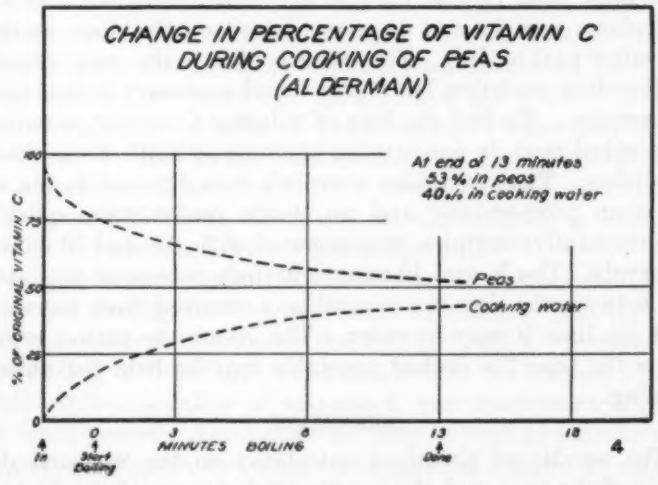


Figure 2





Figs. 3 and 4 These curves were drawn from values obtained by allowing for the vitamin C contained in the samples used for analysis. The amount of cooking water was estimated.

such quantity that, based upon preliminary and concurrent indophenol titrations, each animal received 0.5 or 1.0 mg. of vitamin per day during a period of 14 days. For comparison, other groups received 0.5 and 1.0 mg. of pure vitamin per day, fed in a standard solution from graduated pipettes. Negative controls continued to receive the basal diet only.

The results of the assay, given in table 1, show close agreement in growth response and scurvy scores for the animals receiving test peas, in comparison with those receiving standard vitamin solution. A few animals in each group failed to eat their test food promptly during the first 3 days of the assay period and were therefore discarded.

There was no significant loss in the titration value of the peas during the specified period of storage for the assay. During this period they were held in insulated fiber shipping cases with liberal quantities of dry ice.

2. Losses of vitamin C during the standing of cooked peas. The peas used in this part of the experiment were of the Telephone variety and were purchased on the open market the latter part of July. They were cooked in the same manner as the other varieties, but it was found necessary to boil them 19 minutes. To find the loss of vitamin C during standing, the cooked peas, in one serving amounts (½ cup), were placed on dishes. The vegetables were left thus exposed to the air at room temperature and no doubt cooled very quickly. Representative samples were removed at 5-, 15- and 70-minute intervals. The 5- and 15-minute periods represent the interval between the time the vegetable is removed from the stove and the time it may be eaten. The 70-minute period represents the time the cooked vegetable may be held for animal feeding.

DISCUSSION

The results of titrations calculated on the wet and dry basis of the peas and the results of titrations of the cooking water are presented in tables 2 and 3. The data in tables 2 and 3 are represented graphically by figures 1 and 2. The curves so constructed show the vitamin C content of the raw

and cooked peas, the solution into the cooking water, and the destruction of vitamin C from the peas during the cooking and overcooking. They also show the total loss of vitamin C from the vegetable and the total loss to the cooking water at the 'done' and the 'overdone' stages, the peas being cooked with no interference from sampling. Figures 3 and 4 show the percentage of vitamin C retained in the cooking water and in the peas at the 'done' stage.

The vitamin C content of the raw peas, 0.23 and 0.24 mg. per gram for the Thomas Laxton and the Alderman, respectively, was about the same as the 0.25 mg. per gram (recalculated from biological tests) reported by Eddy, Kohman and Carlsson ('26), but was higher than that reported by Bessey and King ('33) 0.16 mg. per gram (by dye titration). In both of these cases, the product was of uncertain variety and was purchased as a typical market vegetable. The higher content is partly explained by the fact that the peas were picked at the succulent stage and that a minimum length of time elapsed before they were tested. There is also the possibility that the one group of workers may have used varieties of peas lower in vitamin C. In a test of the vitamin C content of eighteen varieties of peas, Mack, Tressler and King ('36) found the Thomas Laxton midway in the group and the Alderman in the lower third. During cooking, the Alderman variety retained vitamin C in the peas to a slightly greater extent than did the Thomas Laxton.

In every case the greatest loss of vitamin C from the vegetable was during the first 2 minutes of cooking (the time it took the water to return to the boiling point). A possible explanation of the large initial loss is that the enzyme which catalyzes the oxidation of vitamin C was inactivated during this short period. Another factor may be that as soon as the oxygen was driven out, there was an atmosphere of steam above the water, and subsequent oxidation was decreased.

The rate of loss of vitamin C after the water came back to the boil was very low and decreased almost to zero.

The large amount of vitamin C dissolved in the cooking water is worthy of notice. This amount increased almost linearly with time. Vinokurov and co-workers ('35) and Halliday and Noble ('36) also found large amounts of vitamin C lost from vegetables to the cooking water. The vitamin C content of the cooking water from peas (0.10 to 0.12 mg. per cubic centimeter) compares favorably with that reported in the literature for tomato juice (0.14 to 0.27 mg. per cubic centimeter).

The total destruction of vitamin C in the Thomas Laxton at the 'done' stage was 10%, 42% being retained in the peas and 48% in the cooking water. The total destruction in the Alderman was 7%, 53% being retained in the peas and 40% in the cooking water.

The slight actual destruction of vitamin C may be partially explained by the low pH of the peas, which remained less than 7 throughout the cooking period. Barron, De Meio and Klemperer ('36) report "ascorbic acid is not autoxidizable in acid and neutral solutions up to pH 7.0."

Upon being overcooked, the peas apparently increased slightly in vitamin C, both on the wet and dry percentage basis. At this point, the peas and the cooking water were both reduced to a small amount, so that the rate of evaporation from the cooking water was very rapid. Another possible factor may have been introduced by the formation of small quantities of non-vitamin dye-reducing substances.

During the standing of the cooked peas, the destruction was very slight, but was greatest during the first 5 minutes, amounting to about 9% on the wet weight basis; at the end of 70 minutes, the destruction had increased to 14% of the vitamin C content of the 'done' peas. It is probable that this small destruction was caused by atmospheric oxygen and occurred largely in the peas on the surface. Consequently, after the preliminary destruction, very little further decomposition took place.

SUMMARY

1. The work shows clearly the value of the cooking water of peas from a nutritive standpoint, since about one-half of the vitamin C passes into it.

2. The greatest rate of loss of vitamin C from the peas occurred during the first 2 minutes of cooking.

3. The question of a few minutes of overcooking or undercooking of peas is not an important factor.

4. The loss of vitamin C during standing of the drained cooked vegetable at room temperature is relatively small and seems to occur during the first few minutes.

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THE RELATION OF VITAMIN G[1] TO THE HATCHABILITY OF HENS' EGGS

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In previous reports (Bethke and Kennard, '28, '30) we pointed out that the ration for the breeding flock must contain the factor or factors carried by milk, fresh grass, or alfalfa leaf meal, in addition to vitamin D, for the production of eggs of high hatchability. Other investigators have also reported that diet is a factor in embryonic development. Byerly, Titus and Ellis ('33 a) found that diets which contained only feed stuffs of vegetable origin yielded eggs in which a relatively large percentage of the embryos died during the second week of incubation, associated with a high incidence of chondrodystrophy. The same authors (Byerly, Titus and Ellis, '33 b) later reported that corn, wheat, ground soy beans, soy bean meal, cottonseed meal, flame-dried menhaden meal and some lots of meat meal are deficient in some substance necessary for the production of eggs capable of supporting embryonic life during the second week of incubation. On the contrary, vacuum dried North Atlantic fish meal, steam-dried crab meal, dried buttermilk, and a combination of dried buttermilk, North Atlantic fish meal and meat meal, and free range were found by these investigators to enable the production of eggs capable of supporting embryonic life through the second week of incubation in normal fashion. The authors state:

[1] Vitamin G(B_2) as used in this paper has reference to the complex and not to any one of the several factors which make up its complexity.

[2] Published with the permission of the director of the Ohio Agricultural Experiment Station.

Evidence as to the nature of the deficiency is somewhat conflicting. The proteins in the deficient concentrates may well have been qualitatively deficient. It is possible that vitamin G, or some one of the less known water soluble vitamins, is concerned, although the authors have thus far been unable to obtain experimental evidence for such a view.

McFarlane, Fulmer and Jukes ('30), in studies of embryonic mortality in chicks found that the source of protein in the ration of the parent stock exerted a marked influence on mortality, which was not associated with the total nitrogen, amino acid, ash, or iron content of the egg. Calvery and Titus ('34) were also unable to find differences in the amino acid makeup of eggs from birds kept on different rations. On the contrary, Titus, Byerly and Ellis ('33) reported data which they interpreted as indicating differences in the crude protein contents of eggs due to the ration fed the hens producing them.

Investigators at the Ontario Agricultural College (Anonymous contribution, '35) found that the feeding of buttermilk, alfalfa leaf meals, and liver extract decreased early embryonic mortality and increased hatchability of the eggs. Halpin, Holmes and Hart ('33) reported that the addition of skimmilk, chopped alfalfa hay, or autoclaved yeast to the ration of laying hens increased the hatchability of the eggs produced by supplying vitamin G. Heiman ('35) also attributed the increased hatchability resulting from the addition of dried skimmilk or dried whey to a cereal-casein ration to the vitamin G content of the milk products added.

The experiments reported in the present paper were undertaken to confirm and extend our earlier studies on the effect of the ration on the ability of eggs produced to sustain good embryonic development. The data presented were obtained during the 4-year period, 1930 to 1934.

EXPERIMENTAL

In all trials White Leghorn yearling hens of known pullet year egg production were used. These were mated with cockerels of the same breed several weeks before the eggs

were saved for incubation purposes. The males in any particular experimental series were rotated three times each week between the different pens, in order to overcome individual differences. All groups of birds were kept indoors in small pens, provided either with straw or wood shavings as litter. The trials were started either the latter part of November or the fore part of December and continued until May or June. Ten females and one male were placed in each pen. The incubation studies were started from 4 to 6 weeks after the hens had been put on their experimental rations. All eggs were set in the same electrically operated incubator, at weekly intervals for a 3- to 5-month period.

The basal rations used in the different trials were of the same general composition except for the source of supplemental protein. They consisted of: Ground wheat, 15.0; wheat bran, 10.0; steamed bone meal, 2.0; fine oyster shells, 4.0; salt, 0.5; cod liver oil, 1.0 to 2.0; either meat scraps, 10.0; menhaden fish meal, 9.0; or casein, 7.5, and yellow corn to make 100 parts. The rations were mixed fresh approximately every 2 weeks, in order to avoid vitamin deterioration. Oyster shells were also kept before the birds in open feeders.

In the first trial a basal ration containing meat scraps was fed. This was supplemented with alfalfa leaf meal, an acidulated water extract of alfalfa leaf meal, dried skim-milk, a milk serum concentrate, and autoclaved yeast. The alfalfa leaf meal replaced an equivalent quantity of wheat bran in the basal mixture. The protein content of all rations was equalized by decreasing proportionately the amount of meat scraps. The yeast was prepared by autoclaving a pure dried bakers' yeast for 6 hours at 15 pounds pressure. The alfalfa extract was made by treating the alfalfa leaf meal with four separate portions of slightly acidulated water and concentrating to a suitable volume before electric fans at room temperature. The milk serum concentrate was obtained from the Dry Milk Company, of Bainbridge, New York. According to the manufacturers, it represents a concentration of the milk serum after the removal of the fat, casein, lacto-albumin, and greater part of the milk sugar and part of the

calcium phosphate, to the consistency of molasses. This concentrate has been shown to be a good source of the vitamin-B complex by Supplee, Dow and Flanigan ('28) and to have growth promoting and leg paralysis preventing properties for chicks by Norris and associates ('30) and Bethke et al. ('31). We have also found it to be a good source of vitamin G(B_2), as determined on rats.

The results of the incubation trials presented in table 1 clearly show that alfalfa leaf meal, dried skimmilk, and autoclaved yeast carry some factor or factors essential for good hatchability, or the proper development of the embryo. In

TABLE 1

Effect of alfalfa leaf meal, water extract of alfalfa, milk and autoclaved yeast on hatchability

PEN	ADDITIONS TO BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	357	90.2	97	30.1
2	5.0% alfalfa leaf meal	459	96.7	298	67.1
3	Water extract of 5.0% alfalfa leaf meal	356	95.5	216	63.5
5	7.5% dried skimmilk	579	95.3	395	71.5
6	2.5% milk serum concentrate	306	93.1	180	63.2
8	1.0% autoclaved yeast	308	96.7	149	50.0
9	3.0% autoclaved yeast	370	89.7	235	70.8

this respect the results with milk and alfalfa confirm our earlier observations (Bethke and Kennard, '28, '30) on this problem. The results obtained with the aqueous extract of alfalfa leaf meal and the milk serum concentrate indicated that the hatchability promoting factor or factors are water soluble and that supplemental protein was probably not concerned in the results secured. The fact that the above products, which gave an increase in hatchability, had been found to be good sources of vitamin G in our laboratory and by other investigators suggested that this factor was essential for embryonic development. Accordingly, our next step was to determine whether liver, which had been shown to be an

excellent source of vitamin G and dried whey, which represented a greater concentration of the water-soluble constituents of milk than dried skimmilk, carried the factor or factors conducive to good hatchability. There was also included one pen on wheat germ and another on wheat germ oil, to determine the effect of additional vitamin E. Although we had experienced no difficulty in obtaining good fertility in the eggs, it was thought advisable to include vitamin E, in view of the more recent experiments on the relation of limited intakes of this factor to nutrition and reproduction. The liver meal, which was prepared from beef liver, and the dried whey replaced equivalent quantities of the meat scraps protein, and the wheat germ an equal amount of wheat bran in

TABLE 2

Effect of liver meal, wheat germ, wheat germ oil and dried whey on hatchability

PEN	ADDITIONS TO BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	297	87.9	21	8.0
2	3% liver meal	708	80.9	486	84.8
4	10% wheat germ	520	91.3	145	30.5
5	2% wheat germ oil	310	90.3	6	2.1
7	5% dried whey	504	84.3	272	64.0

the basal ration. The wheat germ oil was prepared by extraction with anhydrous ethyl ether.

The data presented in table 2 show that the liver meal and dried whey exerted a marked effect on hatchability. The replacement of the wheat bran in the basal ration with pure wheat germ also increased hatchability—indicating that the germ portion of the wheat contained some of the same factor or factors present in alfalfa, yeast, liver and certain milk products. That the favorable response was not associated with vitamin E or other possible factors found in the oil of the germ is shown by the negative results in case of the eggs from pen 5.

Next we desired to determine whether some of the same products which gave an increased hatchability when added to

the meat scraps basal ration, would give similar results when some other source of protein, like menhaden fish meal and an Argentine and a domestic casein, replaced the meat product in the basal ration. The two caseins were used because in studies with chicks and rats the domestic product was found to contain significantly more vitamin G than the Argentine product. It was also desired to obtain information on the stability of the hatchability-promoting factor or factors to autoclaving in an acid and alkaline medium. Several investigators (Williams, Waterman and Gurin, '29; Guha, '31) had

TABLE 3

Effect of alfalfa leaf meal, autoclaved yeast, liver meal and acid and alkaline autoclaved liver meal on hatchability

PEN	MODIFICATIONS OF BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	233	98.3	23	10.0
3	5% alfalfa leaf meal	285	97.5	103	37.0
5	3% untreated liver meal	393	97.7	202	52.9
6	3% acid autoclaved liver meal	434	97.0	240	57.0
7	3% alkaline autoclaved liver meal	194	95.4	8	4.3
8	Argentine casein basal ration	211	95.3	11	5.5
9	Argentine casein 5% autoclaved yeast	336	92.8	182	58.3
10	Argentine casein 3% untreated liver meal	290	97.6	168	59.4
11	Domestic casein basal ration	113	98.2	11	9.9

The basal ration of pens 1 to 7 contained menhaden fish meal, and those of pens 8 to 11 casein.

shown that vitamin G, as determined on rats, was stable in an acid but not in an alkaline, heat-treated medium. A commercial dried beef liver meal was used. The dried meal was made into a thick paste with distilled water and hydrochloric acid and sodium hydroxide added to bring the pH of the respective samples to 3.4 and 11.1 and then autoclaved for 5½ hours at 15 pounds pressure. The pH of the acid-treated meal had risen to 4.05 and the alkaline-treated product had dropped to 9.30 after autoclaving. The heat-treated meals were dried in a hot air oven at 110 to 120°C., ground, and fed as indicated in table 3. The liver meals replaced an equivalent

amount of fish meal protein in the basal ration and the alfalfa leaf meal an equal amount of wheat bran. The yeast fed to pen 9 was autoclaved for 6 hours at 15 pounds pressure at its natural pH.

The incubation data in table 3 again show that the alfalfa leaf meal, liver meal, and autoclaved yeast carried factors essential for good hatchability and that these were stable to autoclaving in an acid medium and labile in an alkaline medium. The fact that the results on the unsupplemented basal rations containing either menhaden fish meal or casein were similar to the results obtained on the unsupplemented meat scraps ration indicates that protein per se was not responsible for the increased hatchability when alfalfa leaf

TABLE 4

Showing that the hatchability factor of dried liver was soluble in 20% alcohol

PEN	ADDITIONS TO CASEIN BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	89	92.1	0	0.0
2	3% dried liver	420	90.9	200	52.3
3	Extract of liver ≏ 3% dried liver	487	87.7	224	52.5
4	Liver residue ≏ 3% dried liver	201	83.1	0	0.0

meal, milk products, liver, and autoclaved yeast were added to the ration. This is further substantiated by the beneficial results obtained with the aqueous extract of alfalfa leaf meal and the milk serum concentrate in the first experiment. Rat and chick experiments also showed that the alkaline autoclaved liver meal was devoid of vitamin G and that the acid heat treatment of the liver product did not affect its vitamin G content.

We also found in experiments with rats and chicks (unpublished) that vitamin G could be extracted from defatted dried liver by 20% alcohol. That the factor or factors in liver essential for good embryonic development were also extracted is shown in the hatchability data in table 4. The extract used in these experiments was prepared by treating the defatted

dried pork liver with several volumes of 20% alcohol, by weight, stirring for 1 to 2 hours and allowing to stand for 12 hours, then filtering and repeating the extraction six times. The extracts were combined and reduced to an appropriate volume before fans at room temperature. The concentrated liquid was then chilled and the precipitate filtered off and added to the extracted residue. The extract and residue were fed at levels comparable to 3% of the untreated dried liver. The extract as fed supplied 90 mg. of protein (NX 6.25) per 100 gm. of ration.

Our next step was to determine whether the hatchability promoting factor of dried whey was soluble in hot 95% alcohol; since Booher ('33) had reported that such an extract contained vitamin G, as determined on rats. We also desired to determine whether vitamin B_4 was a factor in our hatchability results, because Keenan et al. ('33) had reported that the chick requires a factor similar to or identical with this vitamin which they found present in fresh liver and grass. The B_4 concentrate was prepared from fresh pork liver, according to the procedure described by Keenan et al. ('33) through the charcoal stage. The whey extract was prepared by treating the dried product with four separate portions of several volumes of 95% alcohol and refluxing under nitrogen for 2 hours. The alcohol was removed by distillation under vacuum and the residual solution allowed to stand over night in a freezing chamber, to facilitate the removal of the fat and part of the lactose. Both extracts were added to the Argentine casein basal ration. The whey extract was originally added at a level equivalent to 5% of the dried whey, but was increased to 10% 15 weeks after the experiment started. The liver preparation was fed on a 6% dried liver basis. This was replaced at the fifteenth week by 3% of dried liver autoclaved for 5 hours at 15 pounds pressure.

The results, table 5, show that vitamin G is essential for good embryonic development, since the alcoholic extract of dried whey definitely increased the hatchability of the eggs produced. The data also show that the hatchability factor

was not associated with vitamin B_4. It might be assumed that the liver preparation was not fed at a sufficiently high level or that the factor was in part destroyed when mixed with the basal ration to rule out B_4. This does not appear tenable, because the fresh liver dried and autoclaved, which procedure, according to Keenan et al. ('35) destroys this factor, caused a prompt increase in hatchability when it replaced the vitamin B_4 preparation.

TABLE 5

Effect of liver, vitamin B_4, and an alcoholic extract of dried whey on hatchability

PEN	ADDITIONS TO CASEIN BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	152	95.4	24	16.5
2	3% dried pork liver	415	94.2	327	83.6
3	B_4 preparation ≅ 6% dried pork liver	149	90.0	16	11.9
	3% autoclaved dried pork liver replaced B_4 preparation	83	95.2	56	70.9
4	Extract of whey ≅ 5% dried whey	254	91.7	72	30.9
	Extract of whey ≅ 10% dried whey	76	96.0	39	53.4

DISCUSSION

The data definitely show that alfalfa leaf meal, dried skimmilk, dried whey, concentrated milk serum, autoclaved yeast, liver and wheat germ, which are fair to excellent sources of vitamin G as commonly determined on rats, exert a beneficial effect upon the embryonic life of the chick, as measured by hatchability studies. The improvement in hatchability occurred regardless of whether meat scraps, menhaden fish meal, or casein served as the main source of supplemental protein in the ration of the hen. These results, together with the observations that the factor or factors could be extracted from alfalfa leaf meal, dried liver, dried whey, and was present in milk serum practically devoid of protein indicates that the increase in hatchability was not due to protein. The finding that autoclaving of the liver meal in an alkaline medium destroyed the factor affords additional proof that the results

were not due to protein but to vitamin G. Further support that the increased hatchability was caused by vitamin G is found in the observation of Bethke and associates ('36) that eggs which showed high hatchability contained more vitamin G than those which did not hatch well.

Within recent years numerous reports have appeared in the literature which offer evidence that vitamin G, as originally considered, consists of several factors. Since the products we found to increase hatchability have been shown to be good sources of flavins, the data suggest that flavins might be concerned in the investigations reported.

SUMMARY

The inclusion of either alfalfa leaf meal, dried skimmilk, dried whey, autoclaved yeast, dried liver, or wheat germ in the ration of the hen caused an increase in the hatchability of the eggs produced.

It was observed that the increase in hatchability was not associated with protein and that the hatchability-promoting factor or factors were water soluble and extractable from dried liver and dried whey with cold 20% and hot 95% alcohol, respectively.

The factor or factors were destroyed in dried liver by autoclaving in an alkaline medium and were not affected at an acid reaction. The increase in hatchability was not associated with vitamin B_4 or vitamin E (wheat germ oil).

It is concluded that the increase in hatchability noted in the experiments reported was due to vitamin G and that this factor is necessary for the normal embryonic development of the chick.

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THE EFFECT OF THE RATION OF THE HEN ON THE VITAMIN G[1] CONTENT OF EGGS WITH OBSERVATIONS ON THE DISTRIBUTION OF VITAMIN B AND G IN NORMAL EGGS

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FOUR FIGURES

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The literature contains several reports which show that the ration of the hen influences the vitamin content and the hatchability of the eggs produced. Several investigators have shown that the vitamin A and D content of eggs are correlated with the intake of these factors by the bird. Recently Barnum ('35) reported that the vitamin E content of eggs was also related to the vitamin E intake of the hen. The work on vitamin B and G(B_2) has usually been directed toward the relatively potency of eggs in the several factors than toward the effect of the ration of the hen on the eggs. The earlier researches (Cooper, '21; Hoagland and Lee, '24) showed that eggs contained the antineuritic vitamin. Later work by Chick and Roscoe ('29) and Akyroyd and Roscoe ('29) revealed that egg white is devoid of vitamin B(B_1) but is rich in vitamin G while egg yolk contained both factors.

More recently Ellis and associates ('33), in studies on the effect of diet on egg composition, found that the vitamin B content of the diet influenced the amount of this factor in the eggs. The results on vitamin G were not so clear cut. The authors state in part:

[1] Vitamin G(B_2) as used in this paper has reference to the complex and not to any one of the several factors which make up its complexity.

[2] Published with the permission of the director of the Ohio Agricultural Experiment Station.

The results as a whole suggest that the hens' diets did not affect the vitamin G content of the eggs to the extent which has been noted for vitamin B. Lack of vitamin G in the diet apparently did not result in any pronounced drop in the vitamin content of the egg. On the other hand, the inclusion of foods rich in vitamin G in the normal diet used in these experiments tends to enhance the vitamin G content of eggs.

The object of the present studies was twofold: 1) to confirm the observations of Chick and Roscoe ('29) and Aykroyd and Roscoe ('29) on the distribution of vitamin B and G between egg white and egg yolk, and, 2) to determine the effect of the ration of the hen on the vitamin G content of the eggs and to ascertain whether there was a correlation between vitamin G potency and hatchability. We (Bethke, Record and Kennard, '36) had observed that the addition of products rich in vitamin G to the basal ration caused an increase in the per cent of fertile eggs that hatched and that the same products increased growth and prevented the occurrence of a leg disorder in chicks (Bethke, Record and Kennard, '31). These latter observations suggested that vitamin G was essential for the nutrition of the chick, as well as for its embryonic development, and that the high embryonic mortality on certain rations might be related to the amount of vitamin G in the egg.

DISTRIBUTION OF VITAMIN B AND G IN THE EGG

For the determination of the relative distribution of vitamin B and G between egg white and egg yolk we used eggs from hens on a good practical ration of corn, wheat, oats, meat scraps, milk, alfalfa meal and cod liver oil. The whites and yolks were carefully separated by hand and dried at room temperature before electric fans. Weanling rats of our own rearing were used as the experimental animals. These were confined in individual wire cages and fed a basal ration of: Extracted casein, 18; cornstarch, 64; salt mixture, 4; hydrogenated vegetable oil,[3] 10; agar, 2, and cod liver oil, 2. In

[3] Crisco.

determining vitamin B 500 mg. of autoclaved yeast was fed daily and separately to each animal, plus varying amounts of the dried egg products. For vitamin G determination, an 80% alcoholic extract of rice polish, equivalent to 1 gm. of polish, was fed daily and separately as a source of vitamin B in addition to the dried egg products. In each series there were included appropriate negative and positive control groups. Not less than four animals were included in each group.

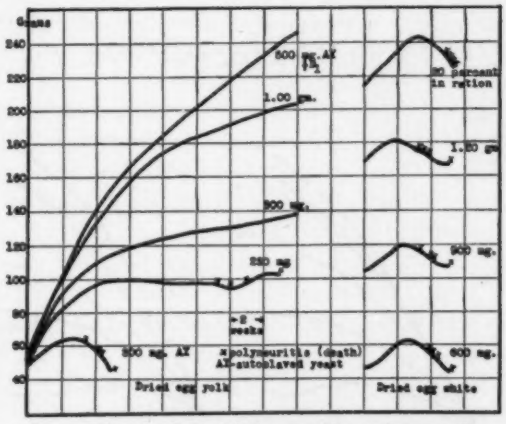

Fig. 1 Vitamin B in dried egg white and egg yolk.

The results obtained on vitamin B are shown in figure 1. It is apparent that egg white is devoid of vitamin B and that the yolk contains all of this factor found in the egg—confirming the observations of Chick and Roscoe ('29) and Aykroyd and Roscoe ('29). The rats on the larger amounts of dried egg white would frequently not consume all of their supplement, so a fourth group was fed a ration in which 20% dried egg white and 10% autoclaved yeast replaced the casein and part of the cornstarch in the basal ration. The performance of this group (fig. 1) was similar to the others fed dried egg white—all animals dying from polyneuritis.

The results on vitamin G, figure 2, show that this factor is found in the egg white and egg yolk—the dried egg white containing from two to two and one-half times as much as the dried yolk. On a fresh or edible basis, the egg yolk would be somewhat more potent in vitamin G than the egg white. All of the animals in the negative control (rice polish extract), 100 mg. dried egg white, 100 and 250 mg. dried egg yolk groups developed skin lesions about the eyes and mouth and frequently these lesions extended to the head, feet and legs. No harmful effects from egg white as reported by Parsons ('31) were noted, except a thinning of the hair coat in two of the animals on the 700 mg. dried egg white level.

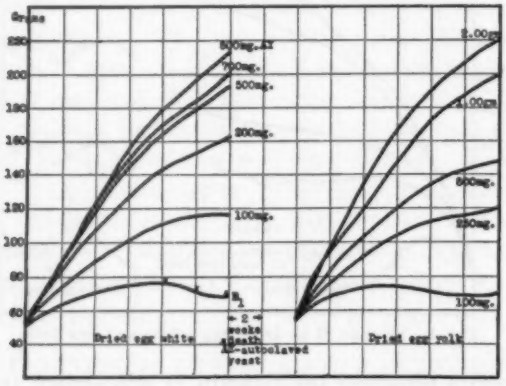

Fig. 2 Vitamin G in dried egg white and egg yolk.

EFFECT OF RATION ON THE VITAMIN G CONTENT OF EGGS

The preliminary studies on the effect of the ration of the hen on the vitamin G content of the eggs produced were carried out only on rats; while in the later investigations both rats and chicks were used. The eggs fed were obtained from hens which were used for hatchability studies. The hens in the different experimental groups had received the same basal ration of yellow corn, wheat, wheat bran, bone meal, oyster shells, salt, and cod liver oil except for source

of supplemental protein or vitamin G supplement for 3 or more months. Further details concerning the hen rations and management procedures are given by Bethke, Record and Kennard ('36).

RAT EXPERIMENTS

Rats of our own rearing, weighing from 40 to 50 gm., were confined in individual wire cages and fed a basal ration composed of: Vitamin-free casein, 18; cornstarch, 64; salt mixture, 4; hydrogenated vegetable oil,[4] 10; agar, 2, and cod liver oil, 2. Vitamin B was supplied in the form of an 80% alcoholic extract of rice polish. An amount equivalent to 500 mg. of rice polish was fed daily to each animal. In the preliminary trials the rats were placed directly on experiment without a depletion period; while in the later experiments the animals were kept on the basal synthetic ration, plus vitamin B for 3 weeks or longer until their weights were stationary or slightly declining over a period of 5 or more days before supplemental feeding was begun. The eggs were fed either dried or in the fresh state diluted with an equal amount of distilled water to facilitate feeding. The dried samples were prepared by thoroughly mixing the yolks and whites and drying in shallow pans at room temperature in a semi-darkened room before electric fans. In each series litter mates were kept on the basal synthetic ration plus vitamin B as negative control groups and in several instances groups receiving either autoclaved yeast or dried pork liver and vitamin B were included for positive control purposes.

The results of the preliminary trial, shown in figure 3, reveal a difference in the vitamin G content of the eggs. The rats fed the eggs produced by hens given the basal ration weighed, on the average, 20 and 48 gm. less, respectively, at the close of the experiment than their litter mates fed the eggs from hens on the same basal ration supplemented with 5% dried whey or 3% liver meal. It is also of interest to note that the growth response of the rats was correlated

[4] Crisco.

with the per cent of fertile eggs that hatched and the vitamin G supplement fed the hens. The eggs produced by hens on the basal ration showed the least vitamin G content and the lowest hatchability (8.0%) whereas the eggs from the liver-fed pen showed the highest vitamin G value and the highest hatchability (84.8%); and those eggs from the dried whey pen occupied an intermediate position with respect to vitamin G content and hatchability (64.0%).

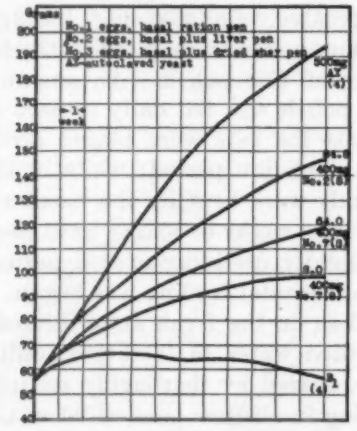

Fig. 3 The vitamin G content of eggs as affected by the addition of 3% dried liver or 5% dried whey to the ration of hens. The figures above the curves represent the per cent of fertile eggs that hatched. The figures in parentheses below the addenda indicate the number of rats in the group.

The foregoing results strongly suggested that the vitamin G content of the egg was associated with embryonic development and led us to investigate further the relation between the hatchability of eggs as affected by the ration and the vitamin G content of the eggs. The rats employed in these studies were first depleted of their vitamin G reserves, as previously indicated, before being placed on experiment. The eggs used for feeding represented random samples from different pens of hens. The eggs in series I were from hens fed either a menhaden fish meal or casein basal ration and the

same rations, respectively, supplemented with 3% of dried pork liver and 5% autoclaved yeast. The series II eggs were from birds on the same basal ration except for different types of fish meals and the basal ration containing menhaden fish meal and 5% commercial liver meal. These eggs were used because the incubation results had previously shown marked differences in the per cent of eggs that hatched. In series III the eggs were produced by hens which received a casein basal ration supplemented either with 3% dried pork liver, 3% autoclaved dried pork liver, or the 95% hot alcoholic extract of dried whey equivalent to 10% of the whey powder. The eggs in this series were fed fresh and dried to determine whether drying at room temperatures in a semi-darkened room affected vitamin G.

The results presented in graphic form in figure 4 show that the vitamin G content of the eggs was affected by the ration of the birds producing them. Without exception, those rations which contained products (dried whey, yeast, liver) which are known to be good to excellent sources of vitamin G yielded eggs that were more potent in vitamin G and that hatched well. It is also evident that there was a correlation between the hatchability of the eggs and their vitamin G content; since almost without exception the higher the per cent of hatch the greater was the vitamin G value. The results obtained on the eggs produced on the different fish meal rations (series II) also show differences in vitamin G content which are in accord with the results of the other egg studies and the hatchability results. The eggs from the menhaden fish meal-liver meal pen were not incubated; however, from results obtained on similar rations previously (series I as well as other trials), we are confident these eggs would have shown good hatchability.

CHICK EXPERIMENTS

The experiments with chicks were carried out to determine whether this species would show similar differences in the vitamin G content of eggs as did the experiments with rats.

The chicks used were White Leghorns hatched from eggs produced by hens fed a good practical ration. They were taken directly from the incubator and placed in brooders provided with wire grid floors and fed a basal ration of ground

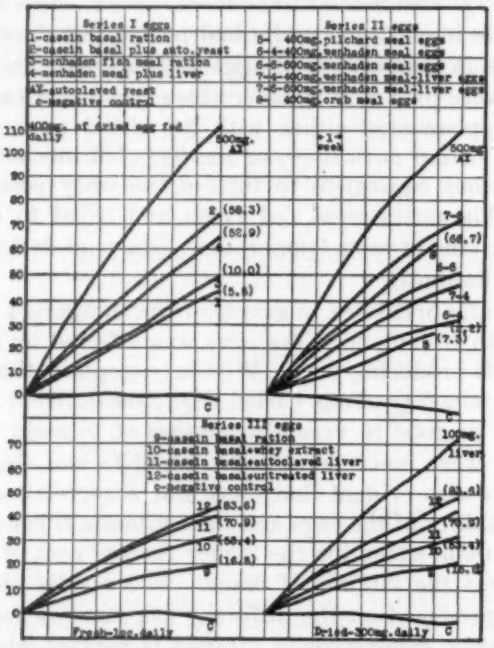

Fig. 4 The effect of the ration of the hen on the vitamin G content of the eggs. The figures in parentheses following the growth curves represent the hatchability (per cent of fertile) of the eggs from the same pens. In series I, five rats were included in the negative and positive control groups and eight in each of the egg groups. Series II contained from six to eight rats on each egg level supplement and four and nine in the positive and negative control groups, respectively. Ten rats were included in each of the groups in series III except the 100 mg. liver group, which contained four.

yellow corn, 58; ground wheat, 20; wheat bran, 5; Argentine casein, 12; steamed bone meal, 3; salt, 1, and cod liver oil, 1. This ration is known (unpublished results) to be deficient in vitamin G for chicks and leads to the development of a leg

disorder, as described by Norris and associates ('30) and Bethke et al. ('31), in a variable percentage of the birds. Beginning with the second week, the shell-free, hard-boiled eggs from the different pens of hens were fed daily for either 6 or 7 weeks.

In the first experiment the eggs from some of the same pens of birds as used in the series II rat studies (fish meal rations) were fed for 6 weeks to groups of twenty-five chicks each. There was also included for comparison one group of chicks on the unsupplemented basal ration. The results, presented in table 1, show that the eggs from the different

TABLE 1

Effect of feeding different fish meal rations to hens on the vitamin G content of the eggs for chicks

EGG SUPPLEMENT	TOTAL BASAL RATION PER CHICK	TOTAL EGG PER CHICK	AVERAGE WEIGHT OF CHICKS	CHICKS SHOWING LEG DISORDER	CHICKS WITH LEG DISORDER RECOVERED	HATCHABILITY OF EGGS[1]
	gm.	gm.	gm.	no.	no.	%
None	556.5	....	173.1	15	3	....
Menhaden fish meal	528.4	215.8	232.5	21	6	2.2
Pilchard fish meal	569.0	216.0	268.2	18	8	7.3
Pilchard fish meal plus 5% dried skimmilk	718.6	226.0	321.1	12	6	46.0
Menhaden fish meal plus 5% dried liver	787.5	225.0	405.8	13	12	....
Crab scrap meal	797.2	216.0	423.4	2	2	66.8

[1] On the basis of fertile eggs.

pens of hens varied in their vitamin G content. The eggs produced on the menhaden and pilchard fish meal rations were less potent in vitamin G, as determined by growth and the prevention of the leg disorder, then eggs produced on similar rations supplemented either with 5% commercial liver meal or 5% dried skimmilk. It is also significant to note that, as in the rat experiments, the higher the hatchability of the eggs the greater was their vitamin G potency. The experiment was, in part, repeated by comparing the eggs from the menhaden fish meal and menhaden fish meal-liver meal pens, with results very similar to those obtained in the above trial.

The second experiment involved a comparison of the eggs from the same pens of hens as in the series III rat trials. The eggs were fed for 7 weeks to groups of eighteen chicks each. There were included for control purposes one group of chicks on the unsupplemented basal ration and another on the basal ration with 3% of dried pork liver. The liver was incorporated in the basal ration when egg feeding was begun, and the protein content adjusted to that of the basal ration by decreasing the amount of casein. The results, table 2, again show that the vitamin G value of the eggs was affected

TABLE 2

Effect of adding vitamin G supplements to the ration of hens on the vitamin G value of the eggs produced for chicks

EGG SUPPLEMENT	TOTAL BASAL RATION PER CHICK	TOTAL EGG PER CHICK	AVERAGE WEIGHT OF CHICKS	CHICKS SHOWING LEG PARALYSIS	CHICKS WITH LEG PARALYSIS RECOVERED	HATCHABILITY OF EGGS[1]
	gm.	gm.	gm.	no.	no.	%
None	577.6	....	184.6	13	0	....
Casein basal ration	681.8	220.0	260.4	14	3	16.5
3% autoclaved dried liver	891.0	203.0	369.2	12	5	70.9
Extract of dried whey ≏ 10%	679.2	204.0	275.6	16	6	53.4
3% dried liver	1002.7	190.0	449.3	2	1	83.6
Basal ration plus 3% dried liver	1393.0	....	574.4	0	0	....

[1] On the basis of fertile eggs.

by the ration of the hen. The inclusion of either autoclaved or untreated dried pork liver in the basal ration of the hens caused the production of eggs with a greater vitamin G content; which was correlated with the hatchability of the eggs. The results obtained with the eggs from the whey extract ration are not in accord with the results procured on the other rations or the rat assay. The chick experiment revealed no difference between the basal ration and basal ration-whey extract eggs; whereas the rat assay and incubation results showed the latter eggs to contain more vitamin G and to hatch better than those produced on the basal ration. A second comparison of the eggs from the basal and whey extract

rations with chicks did not show a significant difference in vitamin G content. We have no explanation to offer for this apparent inconsistency in the results with rats and chicks, unless we were not measuring the same factor in the eggs with the two different species.

Aside from the above exception, the results of the rat and chick assays on the eggs are in good agreement and show that the vitamin G intake of the hen affects the potency of her eggs in this factor. The results also show that embryonic development (hatchability) is closely associated with the vitamin G content of the egg. Apparently vitamin G is essential for embryonic development and unless adequate amounts of this factor are present in the egg, the embryo will die prematurely.

It was observed in preparing the eggs for rat feeding that there was a noticeable difference in the color of the egg white from the different pens. Invariably, the egg whites from those pens which received either autoclaved yeast, dried whey, or liver in their rations showed a distinctly more yellowish green color than the whites from the eggs on the unsupplemented basal rations. This observation suggests that the differences noted in hatchability and in vitamin G content of the eggs might be due to flavin, which has recently been shown to be associated with vitamin G. Experiments are in progress to obtain further information on this point.

SUMMARY

A study of the distribution of vitamins B and G in eggs produced on a good practical ration showed that vitamin B is present in the egg yolk and not in the egg white and that vitamin G is found in both the white and yolk.

Experiments with rats and chicks showed that the ration of the hen affects the vitamin G content of the eggs produced. The inclusion of dried skimmilk, dried whey, autoclaved yeast, dried liver, or certain fish meals in rations low in vitamin G resulted in the production of eggs of increased vitamin G content.

Evidence is presented which shows that embryonic development of the chick (hatchability) is related to the vitamin G content of the eggs.

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THE EFFECT OF POLYNEURITIS IN CHICKS UPON THE IN VIVO RATE OF REMOVAL OF PYRUVATE INJECTED INTRAVENOUSLY [1]

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ONE FIGURE

(Received for publication June 8, 1936)

Our work with chicks, and the investigations of Peters and co-workers with pigeons, indicate that vitamin B(B_1) is concerned with the metabolism of pyruvic acid. Most of the evidence in support of this view is based upon in vitro studies of respiration of tissues from polyneuritic birds. Peters and Thompson ('34) found that pyruvic acid accumulated during the respiration in lactate of brain from polyneuritic pigeons. With normal pigeon brain under similar conditions pyruvic acid did not accumulate. Vitamin B (B_1) added to avitaminous pigeon brain respiring in lactate or pyruvate increased the oxygen uptake. We have shown that in avitaminous chick brain additions of vitamin B had no effect upon respiration in lactate (Sherman and Elvehjem, '36 a, '36 b) but that with pyruvate as substrate, similar additions of vitamin B increased the oxygen uptake of avitaminous cerebrum and kidney.

Although there is good evidence to show that there is a faulty metabolism of pyruvic acid in avitaminous pigeon and chick tissues in vitro, there is insufficient evidence of a failure in the in vivo metabolism of pyruvate in avitaminosis B to

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justify the conclusion that the syndrome of polyneuritis is directly caused by an accumulation of pyruvic acid. Analyses of pigeon brain immediately after the death of the animal have given no indication of a rise in pyruvic acid content (Thompson and Johnson, '35), but Kinnersley and Peters ('29) found an increase in the lactic acid content of avitaminous pigeon brain immediately after death. The failure to detect a rise in the pyruvic acid content of freshly-removed avitaminous tissues may be due, as Thompson and Johnson ('35) point out, to its diffusion from the tissues into the blood stream, since a threefold rise in blood pyruvate of polyneuritic pigeons and more than a twofold rise in the pyruvate content of polyneuritic rats was detected. Pyruvic acid has recently been isolated from the blood of B deficient pigeons (Johnson, '36) in the form of the 2:4 dinitrophenylhydrazone.

Similar studies in our laboratory have given no indication of a rise in either the lactic acid (method of Friedemann and Graeser, '33) or the pyruvic acid (method of Clift and Cook, '32) content of cerebrum or heart in polyneuritic chicks. Nor have we been able to detect a consistent rise in the pyruvate content of avitaminous chick blood. However, in the feces of polyneuritic chicks we have found a considerable rise in bisulphite-binding substances. But before these bisulphite-binding substances can be called pyruvic acid, isolation and characterization studies will be necessary.

Since avitaminous tissues are unable to metabolize pyruvate in contrast to normal tissues, it seems probable that in polyneuritis the excretory organs must have an increased load in order to prevent as long as possible an excessive accumulation of pyruvate in the blood and tissues. Sodium pyruvate was therefore injected intravenously into normal and avitaminous chicks and the rate of its removal was determined. Our results show that injected pyruvate disappears from the blood stream of normal chicks so rapidly that no significant increase could be detected 1 minute after injection of amounts of pyruvate sufficient to produce a great theoretical increase in blood pyruvate. In polyneuritic

chicks, on the other hand, injection of sodium pyruvate produced a sharp increase in blood pyruvate which slowly returned to the normal level.

METHOD

Large normal and polyneuritic chicks weighing from 200 to 400 gm. were used in these studies. Fresh vacuum-distilled pyruvic acid was adjusted to pH 7.3 with NaOH and made into a solution containing 40 mg. pyruvic acid per cubic centimeter. Large veins under each wing were exposed for injection and blood removal. A 27-gauge hypodermic needle attached to a hypodermic syringe containing the solution of sodium pyruvate was inserted into the vein under one wing and a measured amount of solution carefully injected. After a few seconds had elapsed following the injection to allow the blood to carry all of the injected pyruvate from the site of injection back to the heart, the needle was removed. Blood samples were then taken at frequent time intervals from the corresponding vein on the opposite wing with a capillary pipette calibrated to contain 0.2 cc. The blood samples were discharged into centrifuge tubes containing 9.8 cc. of 4% trichloracetic acid. The thin film of blood clinging to the inner wall of the pipette was removed with the trichloracetic acid. Following this technic it was usually possible to obtain the first blood sample from 1 to 2 minutes after the time of injection. The method is somewhat crude, especially in the measurement of the exact time of the injection and the subsequent sampling of blood, since a rapid flow of blood is not always obtained. The time intervals indicated are accurate to within only $\pm\frac{1}{2}$ minute. Pyruvate analyses here recorded were made by the method of Clift and Cook ('32) according to the technic described in an earlier publication (Sherman and Elvehjem, '36 a, '36 b).

For the comparison of the bisulphite-binding capacity of normal and avitaminous chicks, larger samples (2 to 3 cc.) were collected in a beaker from the neck of the decapitated chick and quickly poured into a centrifuge tube containing

trichloracetic acid. The tube was immediately stoppered and weighed. Samples of feces (about 1 gm.) were removed from the cloaca and treated with trichloracetic acid in the same manner as the blood samples. A few analyses were also made on the contents of the large intestine and are included for comparison.

RESULTS

The values obtained for the bisulphite-binding capacity of blood and feces from normal and polyneuritic chicks are given in table 1. There is no apparent increase in the bisulphite-

TABLE 1

Bisulphite-binding substances in the blood and feces of normal and avitaminous chicks. Calculated as milligrams pyruvic acid/100 gm. (1 cc. N/200 I = 0.22 mg. pyruvic acid)

BLOOD		FECES	
Normal	Avitaminous	Normal	Avitaminous
		From large intestine	
11.6	14.2	61	68
19.0	12.2	59	82
17.8	27.8	From cloaca	
15.6	10.9	115	304
13.9	12.1	116	606
	9.3	141	282
		139	264
			288
15.6 Average	14.4	128 Average	349

binding capacity of the blood of the B deficient chick over the normal level. In chicken feces, on the other hand, a rise in bisulphite-binding substances was found in avitaminosis B. In the cloaca, into which the urinary excretion flows, there was nearly a threefold rise in bisulphite-binding substances. A few samples of the contents of the large intestine above the cloaca were analyzed for bisulphite-binding capacity and did not show such a large increase in the polyneuritic chicks.

The effect of the intravenous injection of various amounts of sodium pyruvate upon the blood pyruvate of normal and

avitaminous chicks is given in figure 1. The curves immediately after injection are dotted because no blood samples could be taken during this time, and the immediate effect of the injection upon the pyruvate level of the blood is unknown. With all levels of pyruvate injected a pronounced rise in the blood pyruvate of polyneuritic chicks is apparent in the first sample after the injection (2 or 3 minutes). Succeeding samples show that the high initial pyruvate level drops off rapidly at first and then slowly approaches the normal level.

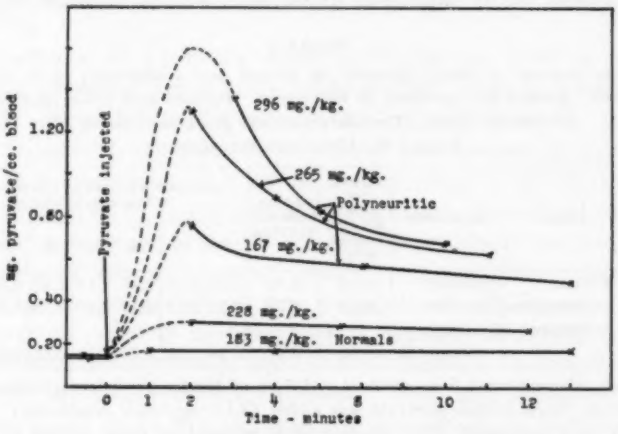

Figure 1

Intravenous injections of comparable levels of pyruvate into normal chicks produce only a very slight rise in blood pyruvate. The first sample was taken as soon as possible after the injection in order to detect, if possible, an increase of short duration, but none was found.[2]

Such a rapid removal of pyruvate from normal blood suggested the possibility that blood itself might be able to metabolize pyruvic acid. Normal blood and avitaminous blood with and without added vitamin B were tested in vitro for their ability to metabolize pyruvate. Sodium pyruvate

[2] We wish to thank Mr. V. R. Potter for his assistance in making injections.

was added to Ringer's phosphate + pyrophosphate buffer pH 7.3, and made isotonic by the addition of NaCl. This solution containing 4.14 mg. pyruvic acid per cubic centimeter was mixed with equal volumes of the three samples of blood and incubated for 1 hour at 37°C. and vigorously shaken with air every 5 minutes. During this period of incubation 1 cc. samples were removed from each tube at frequent intervals for pyruvate analysis. Reference to table 2 shows that pyruvic acid is not metabolized by either normal blood, avitaminous blood, or avitaminous blood with added vitamin B.

TABLE 2

Aerobic removal of added pyruvate by normal and avitaminous chick blood in vitro. Results are expressed in milligrams. Pyruvic acid/cubic centimeter of diluted blood. Time intervals are given in minutes after mixing the blood with the pyruvate

TUBE	BLOOD	ADDITIONS	BLANK ON BLOOD MG./CC. WHOLE BLOOD	INITIAL CONCENTRATION	CONCENTRATIONS AT LATER TIME INTERVALS, MINUTES						
					6	12	18	24	33	41	56
1	Normal	Pyruvate	0.15	2.12	2.12	2.12	2.16	2.14	2.12	2.14	2.12
2	Avitaminous	Pyruvate	0.13	2.13	2.16	2.16	2.17	2.20	2.18	2.19	2.18
3	Avitaminous	Pyruvate + vitamin B	0.13	2.13	2.18	2.22	2.20	2.18	2.22	2.20	2.18

Each tube contained 5 cc. of blood and 5 cc. of Ringer phosphate-pyrophosphate buffer to which sodium pyruvate was added (4.14 mg./cubic centimeter). Five-tenths cubic centimeter (12.5 gamma) of B replaced an equal volume of buffer in tube 3.

DISCUSSION

Our results indicate that in chicks polyneuritis does not produce a rise in blood bisulphite-binding substances. The values for normal and polyneuritic chicks are comparable to results reported by Thompson and Johnson ('35) for avitaminous pigeon blood. But they found the normal level in pigeon blood to be only one-third the avitaminous level. It appears that the normal level of bisulphite-binding substances in the blood of chicks on a natural grain ration so closely approaches the renal threshold that any further increase arising from a faulty metabolism of pyruvic acid in the

avitaminous tissues results in its excretion since we have found a threefold increase in the excreta of polyneuritic chicks. As yet we have not attempted to characterize the compounds present in normal and avitaminous chick blood and feces responsible for their bisulphite-binding capacity. The results of Thompson and Johnson ('35) indicate that the bisulphite-binding compounds of normal pigeon blood are not pyruvic acid. Similar studies upon the nature of the bisulphite-binding substances of chick blood and feces should be undertaken to determine if there is a change in polyneuritis.

Injections of pyruvate show that pyruvic acid is more rapidly removed from normal blood than from avitaminous blood. Since this difference in the rate of removal cannot be attributed to changes in the blood metabolism of pyruvic acid in polyneuritis, it must be due to either a lowered rate of excretion or to faulty tissue metabolism. In normal chicks injected pyruvate is rapidly removed from blood because two channels of removal are at hand: 1) Absorption from the blood by the tissues where it is metabolized. 2) Passage into the excreta. But in avitaminosis B, because of faulty tissue metabolism of pyruvate, the rate of removal is largely dependent upon the excretory process. The time necessary for all the blood to be filtered through the kidneys is sufficiently great to permit the detection of a rise in blood pyruvate following its injection.

SUMMARY

1. The bisulphite-binding substances of normal and B avitaminous chick blood and feces have been investigated. Intravenous injections of sodium pyruvate were made in normal and polyneuritic chicks, and its rate of removal from the blood determined.

2. There is no rise in the bisulphite-binding substances of the blood of chicks in avitaminosis B, but the excreta of polyneuritic chicks contain increased amounts of bisulphite-binding substances.

3. Pyruvate injected intravenously is very rapidly removed from the blood of normal chicks. A slow rate of removal was found in polyneuritic chicks.

4. These phenomena are consistent with the view that in polyneuritis there is a failure in the tissue metabolism of pyruvic acid.

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